Functional and cell-specific regulation of the Brn-3b transcription factor by microRNAs

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I, Mattia Calissano, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

The wheel was invented to work less and to have more time exploring the world. Therefore, thoughtfulness, curiosity and a bit of laziness are a stronger propeller of human evolution than mere ambition.

Let's slow down so we can get there faster.
Read somewhere in London

Acknowledgments

I would like to thank the support and supervision of Professor David Latchman without whom this work could have not been possible. In the years spent in his laboratory he gave me all the support and freedom to pursue my interests whilst keeping all my projects on track.

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Abstract

Post-transcriptional gene regulation is a very powerful and evolutionary conserved mechanism which allows cells to finely modulate gene expression by acting at the level of their mRNAs. In this thesis the post-transcriptional regulation of the Brn-3b transcription factor was analyzed as a case study. This protein belongs to the POUIV family and to the homeobox super-family of transcription factors which are involved in neuronal differentiation and body patterning.

The data presented in this thesis show that regulatory sequences contained in the 3'UTR of Brn-3b mediate the degradation of its mRNA in some neuronal cell lines. Furthermore, the regions regulating this effect have been characterized and two different microRNAs, very short RNA molecules which are key players in the post-transcriptional regulation of mRNAs, have been found to be directly involved in regulating the levels of Brn-3b.

As Brn-3b plays an essential role in the survival of retinal ganglion cells, it was also investigated whether its mRNA is similarly regulated in a retinal ganglion cell line (RGC-5) as it occurs in other neuronal cell lines. Intriguingly, possibly due to its pro-survival role, Brn-3b is protected from degradation by microRNAs in RGC-5 cells in contrast to its fate in other cell types. Furthermore, one of the two microRNAs involved in its regulation is not expressed in the RGC-5 cell line, paralleling its expression profile in primary RGCs.

Following a brief analysis concerning the possible regulation of Brn-3b in primary retinal ganglion cells, the possible general role of post transcriptional gene regulation was assessed by evaluating the role of Dicer in the differentiation of primary

retinal ganglion cells. This showed that Dicer, one of the key enzymes in the production of microRNAs, might be involved in the modulation of neuronal differentiation markers and might thus have a direct role in retinal ganglion cell differentiation.

Overall this thesis provides evidences that post-transcriptional gene regulation is a mechanism which might modulate the levels of mRNA molecules depending on whether the encoded protein has a pro-survival role. Furthermore, preliminary evidences suggest that Dicer, a key enzyme in the production of microRNAs, might have a role in the modulation of retinal ganglion cells differentiation markers.

Publications derived from this work

Mattia Calissano, James Diss, David Latchman “Post-transcriptional regulation of the Brn-3b transcription factor in differentiating neuroblastoma cells” FEBS letters, 2007, vol. 581 (13), 2490-2496.

Mattia Calissano, David Latchman “The pro-survival Brn-3b transcription factor is protected from microRNA mediated degradation in retinal ganglion cells.” Molecular and cellular biology, submitted. 2009

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Abbreviations

3'UTR	3' Untranslated region
AD	Activation domain
ADs	Alzheimer's disease
bp	Base pairs
Brn-3*	Brn-3 transcription factors
BSA	Bovine serum albumin
cDNA	Copy DNA
cBrn-3b	Chicken Brn-3b
cDicer	Chicken Dicer
CHS	Chalcone synthase
CLL	Chronic lymphocytic leukemia
DAPI	4', 6-diamidino-2-phenylindole
DBD	DNA binding domain
DMSO	Dymethyl-Sulfoxide
dNTP	Deoxy nucleotide triphosphate
DRG	Dorsal root ganglia
EDTA	Ethylenediaminetetraacetic acid
FCS	Foetal calf serum
FITC	Fluorescein isothiocyanate,
FXS	Fragile X syndrome
GFP	Green fluorescent protein

GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
HBSS	Hanks Balanced Salt Solution
HRP	Horseradish peroxidise
KCl	Potassium Chloride
LB	Luria broth
$MgCl_2$	Magnesium Chloride
NGF	Nerve growth Factor
PBS	Phosphate buffer saline
PCR	Polymerase chain reaction
PD	Parkinson disease
PFA	Paraformaldheyde
PLL	Poly L-Lysine
PTGR	Post-transcriptional gene regulation
RFP	Red Fluorescent Protein
RGC	Retinal ganglion cell
SDS	Sodium dodecyl sulphate
SNP	Single nucleotide polymorphism
SS	Staurosporine
TF	Transcription factor
VEGF	Vascular endothelial growth factor

Chapter 1

Introduction

1.1 Mechanisms involved in gene regulation

Transcription is the biological process by which the genetic material is copied into RNA (Berg et al. 1964). This process, as many other biological phenomena, is subjected to a high and complex degree of regulation (Wolberger 1999; Li et al. 2010).

Simple unicellular organisms such as E. *coli*, have found simple but efficient and elegant ways of controlling and regulating the expression of their genome (Grainger et al. 2009). For example, via a system called the *Lac* operon (Jacob et al. 1961), *E. coli* synthesize the enzymes required to metabolize lactose only when lactose is present in the growing medium (Jacob et al. 1961). Multicellular organisms differentiate the expression pattern and levels of specific genes to best suit the needs of a specific group of cells (Goldberger 1974) .

Several mechanisms are involved in the control of the phenotypic expression of the genome of every biota. These can be schematically divided in 1) mechanisms controlling the transcription of the genomic material (Losick R. 1976; Platt 1986), 2) mechanisms controlling the translation of mRNA into protein (Platt 1986; Beyer et al. 1988; Kruys et al. 1988) and 3) mechanisms involved in protein turnover and stability (Garlick et al. 1976; Schrader et al. 2009). All these mechanisms will be described and analyzed in the following paragraphs.

1.1.1 Transcription and transcription factors

Transcription is the first step in the expression of the genome and is a process controlled at different levels (Losick R. 1976; Platt 1986). Nuclear DNA, for instance, is

tightly bound to several classes of proteins which regulate its duplication, transcriptional state and protect it from degradation (Das et al. 1964; Falbo et al. 2010; Unnikrishnan et al. 2010). Depending on the staining properties which reflect the tightness of DNA packaging with proteins, chromatin (from the Greek for 'colour' (Flemming 1882) can be found in two forms which, according to the state of their condensation were originally termed heterochromatin and euchromatin (Heitz 1929). The former is generally found at the periphery of the nucleus (Andrulis et al. 1998) and refers to a type of tightly packed chromatin, while the latter, euchromatin, is a form of "loose" DNA packaging (Richards et al. 2002; Richards 2008). The two forms have been historically associated with different transcriptional activity, whereby heterochromatin is transcriptionally inactive (Andrulis et al. 1998; Tham et al. 2001; Mondoux et al. 2007) and euchromatin active (Hsu 1962). This schematic scenario has been superseded by evidences which show that heterochromatin can play a very important role in chromosomal organization and inheritance (Gatti et al. 1992; Dernburg et al. 1996; Coulthard et al. 2003) and its remodelling has an important role during the early stages of mouse embryo development (Santos et al. 2002; Santos et al. 2010).

In certain instances, same regions of DNA can be in either a heterochromatic or euchromatic state (Lyon 1962). This is exemplified by the phenomenon known as X-chromosome inactivation (Lyon 1962) whereby of the two X-chromosomes contained in a cell of a female mammal, one of the two is rendered inactive by the acquisition of heterochromatic state while the other is transcribed (Ohno et al. 1959; Lyon 1961). A gene, Xist, which is expressed only by the chromosome which will be inactivated (Brown et al. 1991), is at the basis of this type of control (Brown et al. 1991). Following its

transcription, *Xist* RNA "covers" the X-chromosome from which it was transcribed and inactivates it (Penny et al. 1996; Panning et al. 1997). The mechanism that selects whether the maternal or paternal X-chromosome is to become inactive is currently considered to be random (Okamoto et al. 2004), but once this state is acquired it will be transmitted to daughter cells (Okamoto et al. 2004).

A further level of transcriptional regulation is based on the assembly of the transcription complex, or pre-initiation complex, on the promoter of genes (Lee et al. 2000; Latchman 2002). Amongst other proteins, TFs play an essential and highly regulated role in the assembly and function of the pre-initiation complex (Lee et al. 2000). This is best exemplified by a description of some of the known events regulating the activity of TF's.

Monomeric inactive TFs, for instance, can become active upon their dimerization (Latchman 2002). The STAT1 transcription factor is inactive in its monomeric form but, upon the activation by the JAK pathway dimerizes (Chen et al. 1998) and, upon its binding to nucleolin, a cytoplasmic-nuclear shuttling protein, it relocates from the cytoplasm to the nucleus (Jerke et al. 2009).

TFs can be post-translationally phosphorylated or glycosylated, a chemical modification that can have a profound effect on their activity (Bohmann 1990; Papavassiliou et al. 1992). The phosphorylation of the cAMP-response-element-binding (CREB) transcription factor by protein kinase-A, for instance, alleviates the repressive activity exerted by CREB on the c-jun promoter thereby stimulating its expression (Lamph et al. 1990). The importance of correct phosphorylation of TFs in cellular physiology can be also exemplified by the observation that individuals harbouring a

mutation at threonine-58 of c-myc show an increased incidence in Burkitt lymphomas (Niklinski et al. 2000). Threonine-58 of c-myc is also subjected to glycosylation, a different post-translational modification which possibly affects the sub-cellular localization of this TF (Chou et al. 1995; Chou et al. 1995). Glycosylation can also play a direct role in the transactivation activity of a TF as exemplified by the 3-5 fold increase in activity of the SP1 transcription factor following its glycosylation (Jackson et al. 1988). Conversely, a reduced glycosylation state of this transcription factor is associated to its increased degradation (Han et al. 1997).

The activity of TFs can be also modulated by their binding to specific ligands which can mediate their relocation from one sub-cellular compartment to another. This can be exemplified by the relocation from the cytoplasm into the nucleus of the estrogen receptor (ER) following its binding to estrogen (Hansen et al. 1986; Murdoch et al. 1991) or by the relocation of the vitamin D receptor (VDR) following its binding to Vitamin D (Luo et al. 1994). Many of the aforementioned mechanisms can combine in different ways to affect the modulation of a transcription factor (Orti et al. 1992) .

Although TFs often exert their activity following their binding to specific DNA target sequences, this is not always the case. The COUP and S300-II, for instance, are two transcription factors which are both required for the activation of the ovoalbumin gene in *Drosophila melanogaster* (Tsai et al. 1987). While COUP binds a target sequence on the ovoalbumin promoter, S300-II exerts its function by stabilizing the COPU-ovoalbumin promoter complex by interacting with COUP rather than to specific DNA sequences (Tsai et al. 1987). Alternatively, the binding of a transcription factor to multiple sites of the same promoter has a co-operative effect on its activation. This is the

case, for instance of the co-operative effect exerted by the binding of HSTF on three sites of the *HSP70* promoter (Topol et al. 1985).

The activity, function and localization of TFs and of other proteins associated with the transcription complex can be modulated by more events which, although will not be explored further in this thesis, play nonetheless an essential role in the regulation of gene expression at the transcriptional level (Jerke et al. 2009).

The classification of transcription factors is mainly based on specific characteristics shared by its members such as the presence of conserved domains and the DNA consensus sequences they bind to (Stegmaier et al. 2004; Homsi et al. 2009). As an example, some of the most common TF families are reported in the table below (table 1.0).

TF class	General characteristics
Zn-finger	Four Cysteines/Histidines binding a zinc atom. One or more of these "fingers" binds the major groove of DNA.
Homeodomain	Common in TF involved in body patterning. Mutation in these TF cause the replacement of one body structure with another.
Helix-loop-Helix	Two alpha helices composed of 20 aa joined by a loop.
Basic Leucine zipper	Hydrophobic dimerization structure characterized by the abundance of the aminoacid Leucine.
Paired Box	A conserved domain of 128 aa that controls embryonic development.
T-box	A conserved 200 aa long domain found in TF that generates lineage diversity in the developing embryo.

Table 1.0: Common transcription factor families.

Each family of TFs is made of several members and sub-groups thus providing a large numbers of similar yet unique variations in their ability to bind specific DNA sequences.

1.1.2 Post-transcriptional gene regulation

Another mechanism involved in the modulation of gene expression is based on the control of the splicing, intra-cellular localization, translation rate and turnover of mRNA molecules (Salditt-Georgieff et al. 1980; Beyer et al. 1988; Rasmussen et al. 1993; Millevoi et al. 2009; Schoenberg et al. 2009; Pawlicki et al. 2010).

The major steps in mRNA biogenesis, mRNA transcription, 5' capping, splicing and polyadenylation occur all at the same time in the nucleus (Salditt-Georgieff et al. 1980; Beyer et al. 1988; Rasmussen et al. 1993; Millevoi et al. 2009; Schoenberg et al. 2009; Pawlicki et al. 2010). Nascent mRNA molecules are bound by a group of proteins called hnRNP (heterogeneous nuclear riboprotein) (Choi et al. 1984) which are involved in several of mRNA maturation events (Krecic et al. 1999; Shyu et al. 2000). As mentioned above, nascent mRNA molecules undergo a process named splicing (Berget et al. 1977; Beyer et al. 1988) which mediates the re-arrangement of the coding regions (exons) of the nascent mRNA molecule by the excision of specific non-coding parts (introns) (Berget et al. 1977; MacCumber et al. 1984; Blanchette et al. 2009). The differential re-arrangement of exons leads to the formation of mature mRNA molecules which encode for different proteins in a process called alternative splicing (Early et al. 1980; Perlman et al. 1982). This was initially discovered following the finding that membrane bound and secreted antibodies, which have slightly different molecular weight

due to a difference in their μ heavy chain, are actually encoded by the same mRNA (Alt et al. 1980; Early et al. 1980). The alternative splicing of this mRNA produces two different antibodies (Alt et al. 1980; Early et al. 1980). It has been recently evaluated that circa 95% of nascent mRNAs with more than one exon can yield mRNAs which, by a process of alternative splicing will produce different polypeptides (Pan et al. 2008; Wang et al. 2008; Pan et al. 2009).

Although splicing is generally regulated by the splicesome, a ribonucleo-protein complex involved in splicing (Grabowski et al. 1985), occasionally introns can self-splice from an mRNA molecule (Waring et al. 1984; van der Horst et al. 1985). This characteristic resembles that of rybozymes (Pace et al. 1985; Johnston et al. 2001), i.e. RNA molecules with enzymatic properties present in both eukaryotes and prokaryotes (Serganov et al. 2007).

Translation of mRNA molecules can also be efficiently controlled by regulating their intra-cellular localization (St Johnston 1995; St Johnston 2005) as exemplified, for instance, by the sub-cellular localization of mRNAs in Drosophila melanogaster oocytes (Gillespie et al. 1995; Becalska et al. 2009) or in the denditres and axons of mammalian neurons (Aakalu et al. 2001; Eberwine et al. 2001; Brittis et al. 2002; Verma et al. 2005; Sotelo-Silveira et al. 2006; Yoon et al. 2009).

RNA editing (Lewin 1983; Golden et al. 2008) is a yet another mechanism involved in regulating the message encoded by a mRNA molecule and which is based on the specific modification of some of its cytidine bases into uridine ones (or adenosine into inosine) by an enzymatic reaction (Chen et al. 1990; Davidson 2002). This mechanism leads to the modification of mRNA codon(s) and to the subsequent production of a

different protein from the one encoded by the gene as exemplified by the modification of the calcium permeability of a subunit of the AMPA receptor in the central nervous system of the rat following the editing of the its mRNA (Lomeli et al. 1994).

The translation rate and turnover of mRNA(s) is a further mechanism to efficiently modulate gene expression (Lam et al. 2001; Serikawa et al. 2003; Brockmann R. 2007). Although the process is still being elucidated, it is known that *in cis* sequences contained within the 3' untranslated region (3'UTR) of mRNAs, such as the AU-rich regions (AREs), are used as signals to regulate mRNA half-life (Kruys et al. 1988; Barreau et al. 2005). An actively translated mRNA is normally protected from degradation by mRNA-binding proteins such as the eukaryotic elongation factors elF-4E and elF-4G and poly-A proteins (Andrei et al. 2005). These act in concert to block the decapping and poly-A tail shortening that allow nucleases to degrade the mRNA molecule (Shaw et al. 1986; Kruys et al. 1988). Furthermore, other RNA molecules such as siRNA or microRNAs can mediate the degradation of an mRNA by binding specific target sequences commonly localized on its 3'UTR (Elbashir et al. 2001; Lai 2002). These short RNA molecules provide the target specificity to a ribonucleoproteic complex named RISC (RNA Induced Silencing Complex) (Bernstein et al. 2001; Caudy et al. 2002), which, via specific nucleases mediates the degradation or translation inhibition of a target mRNA. This last mechanism is part of the focus of this thesis and will be discussed in more detail from paragraph 1.2.

1.1.3 Protein turnover

A further mechanism that is involved in the phenotypic expression of DNA is based on the modulation of protein stability and turnover (Garlick et al. 1976; Schrader et al. 2009). An intra-cellular quality control mechanism, for instance, 'scans' proteins from their synthesis to their folding into secondary and tertiary structures (Marquardt et al. 1992; Roth et al. 2008). Misfolded and thus potentially dangerous proteins are removed from the cellular pool and directed towards degradation (Tanaka et al. 1988; Shimbara et al. 1992; Goldberg 2003). A misfolded protein not only might be impaired in its function but it might also act as dominant negative and inhibit the function of other proteins it might bind to (Thompson et al. 2002; Leandro et al. 2008). A mutated form of Hsc4P, for instance, interferes with the intra-cellular function of its wild type counterpart leading to an accumulation of misfolded proteins in the cytoplasm which in turn causes cell death in *Drosophila melanogaster* (Elefant et al. 1999; Thompson et al. 2002; Leandro et al. 2008). In certain occasions, and by mechanisms which are still not fully understood, this event can cause tissue and organ specific diseases (Thompson et al. 2002). This scenario is especially important as it is at the basis of several diseases affecting the central nervous system including Alzheimer Disease, Prion related diseases, Tauopathies and Parkinson Disease (Pan et al. 1993; Aguzzi et al. 2009; Clavaguera et al. 2009; Frost et al. 2009; Ren et al. 2009). The aminoacid composition of proteins also plays a role in controlling their degradation (Schrader et al. 2009). Proteins rich in the aminoacids proline, glutamic acid, serine and threonine (PEST) are generally rapidly degraded (Rogers et al. 1986). Furthermore, protein turnover rate can be modified following changed intra-cellular circumstances. Due to its protective and anti-apoptotic function, for example, the half-life

of the protein p53 is increased 5-10 fold in mouse keratinocyte irradiated with UV-B compared to non-irradiated ones (Liu et al. 1994).

1.2 Post-transcriptional regulation by small RNAs: an historical outline

Post-transcriptional gene regulation was discovered in the early 1990's while attempting to increase the amount of anthocyanin pigment produced by petunia flowers (Napoli et al. 1990). Napoli and his collaborators discovered that *circa* 40% of plants that expressed exogenously-transfected extra copies of the chalcone synthase (*Chs*) gene, a key enzyme in the production of the pigment, produced completely white flowers (Napoli et al. 1990). Other transgenic plants developed flowers with an intricate pattern of white interspersed with pigment (Napoli et al. 1990). The authors measured the levels of *Chs* mRNA in the white flowers and found that both the exogenous and the endogenous *Chs* mRNAs were dramatically reduced, a phenomenon they named co-suppression (Napoli et al. 1990). It was also realized that the white phenotype co-segregated with the exogenous construct (Napoli et al. 1990), i.e. it reverted to wild-type phenotype as soon as the transgene was lost. This suggested that the expression of the exogenous construct triggered intra-cellular events leading to the suppression of *Chs* expression (Napoli et al. 1990). The phenomenon was interpreted as due to the repression of the exogenous and endogenous CHS gene by methylation (Napoli et al. 1990). Beside the introduction of point mutations in duplicated genes in the fungus *N. crassa* (Selker et al. 1988) in a process called RIP (repeat induced point mutations), the study by Napoli and colluegues

was the first detailed observation that increased gene copy number in an organism could lead to the suppression of their expression rather than to their increase.

Several years later it was discovered that, in the nematode *C. elegans*, the gene *lin-4* produces two transcripts of 61 and 22 nt long, which, although being too short to encode for a protein, were nonetheless directly involved in negatively regulating the levels of Lin-14 protein (Lee et al. 1993). *Lin-4*, and a few years later *Lin-7*(Abbott et al. 2005), were the first of a series of short RNA molecules eventually named microRNA or miRNA which are directly involved in gene regulation (Reinhart et al. 2000). Subsequently it was discovered that the injection of a dsRNA targeting a specific gene into C. *elegans*, resulted in a sustained and non-stoichiometric reduction of the levels of its mRNA (Fire et al. 1998). Although, once more, the phenomenon was interpreted as due to regulation at the genomic level (Fire et al. 1998), it was also suggested that it might occur at the RNA level and it might mimic a physiological phenomenon which was named RNAi, RNA inhibition (Fire et al. 1998).

Meanwhile, the investigation of the phenomenon of co-suppression in plants led to the discovery that the degradation of the exogenous and endogenous mRNAs of the same gene was associated with the production of very short RNA molecules, roughly 25 nucleotides long (Hamilton et al. 1999) which were then named siRNA few years later (Elbashir et al. 2001). Shortly afterwards it was eventually realized that co-suppresion in plants and RNAi in C. *elegans* were phenomena part of a similar mechanism and that microRNAs and siRNAs are its key-players (Hammond et al. 2000; Lagos-Quintana et al. 2001; Lau et al. 2001; Lee et al. 2001).

The discovery of microRNAs has added a new level to our understanding of the mechanisms involved in post-transcriptional gene regulation. Since siRNA were initially discovered during transgene and virus mediated manipulation of gene copy number in plants (Waterhouse et al. 1998; Hamilton et al. 1999) it was initially assumed that these molecules played a role in the defence of the genome (Waterhouse et al. 1998). Intriguingly siRNA have now been found as also originating from centromeres, repeat RNA transcripts and transposons (Volpe et al. 2002; Aravin et al. 2003; Sijen et al. 2003). SiRNAs do not thus seem confined to an exogenous origin and other are apparently the actual features that distinguish siRNA form microRNA. For instance, siRNA are generally slightly shorter than microRNA (circa 21 vs. 22-23 nucleotide long) (Hamilton et al. 1999; Caplen et al. 2001; Elbashir et al. 2001). Secondly, the microRNA pathway initiates in the nucleus (Lee et al. 2003). Thirdly, siRNAs are produced in the cytoplasm by Dicer-2, (a protein whose role will be described in detail in the next paragraphs) while microRNAs are processed by Dicer-1 (Lee et al. 2004; Okamura et al. 2004). Further differences can be identified between siRNAs and microRNAs which, eluding the scope of this thesis, will not be further analyzed but have been extensively investigated and reviewed (Tang 2005; Golden et al. 2008; Shabalina et al. 2008; Carthew et al. 2009).

Beside RNAi, long dsRNA can trigger an interferon-based response pathway which involves the activation of PKR, a protein kinase involved in RNA degradation (Thomis et al. 1993).

1.2.1 microRNAs: a general overview

As mentioned before, microRNAs or miRNAs are 22-23 nucleotide long single stranded RNA molecules which are produced from long precursor transcripts (1-2 Kb) from within the genome of virtually every organism studied so far (Finnegan et al. 2003; Allen et al. 2004; Altuvia et al. 2005; Onishi et al. 2005; Smalheiser et al. 2005; Ying et al. 2005; Zhang 2005). MiRNA transcripts can derive from intergenic regions where they are synthesized from their own promoters by RNA-polymerase II (Lee et al. 2004; Corcoran et al. 2009; Reddy et al. 2009) or from within intronic sequences of genes, in which case they are transcribed following the gene's own promoter (Ozsolak et al. 2008; Corcoran et al. 2009). According to currently available data, a slight majority of intergenic vs. intragenic miRNA transcripts are found in every biota studied so far and very little is known about the regulation of miRNA promoters (Griffiths-Jones 2006; Griffiths-Jones et al. 2006; Kim et al. 2007). MicroRNA transcripts are modified like any coding mRNA so that their 5' end is 'capped' with methyl-guanosine and a poly(A)-tail added to it's 3' end (Cai et al. 2004).

1.2.2 From pri-mirRNA to pre-miRNA

Following their synthesis in the nucleus, pri-miRNA are incorporated in a structure named the microprocessor (Denli et al. 2004). This is mainly composed of two proteins: Drosha, an RNAse III enzyme and its cofactor DGCR8 (known as Pasha in *D. melanogaster* and *C. elegans*), which has a double-stranded RNA binding domain (Lee

et al. 2003; Denli et al. 2004; Gregory et al. 2004; Han et al. 2004; Landthaler et al. 2004). RNAseIII enzymes are proteins capable to bind and cleave double stranded RNA (Carmell et al. 2004) and are classified in three classes according to specific domains and functions. Drosha belongs to class II (Filippov et al. 2000), some prokaryotic and fungi RNAseIII to class I (Nicholson 1999) and Dicer, another RNAse involved in the maturation of microRNAs, belongs to class III (Bernstein et al. 2001). The selection mechanism(s) employed by the microprocessor to choose a specific RNA molecule as substrate is not fully known (Gaudin et al. 2006; Han et al. 2006). It has been shown (Han et al. 2006) that a 'typical' pri-miRNA molecule is characterized by one or more loops preceded by a stem of roughly three helical turns and which is surrounded at both ends by less essential segments (Han et al. 2006). Drosha cleaves the RNA circa two helices away from the loop leaving a 70 nucleotide long stem-and-loop structure, the pre-miRNA (Figure 1.0) (Han et al. 2006).

Occasionally microRNAs can arise from spliced introns (Ruby et al. 2007) which are called mirtrons (Ruby et al. 2007) and access the microRNA biogenesis pathway bypassing the first maturation step mediated by Drosha (Berezikov et al. 2007; Okamura et al. 2007; Ruby et al. 2007).

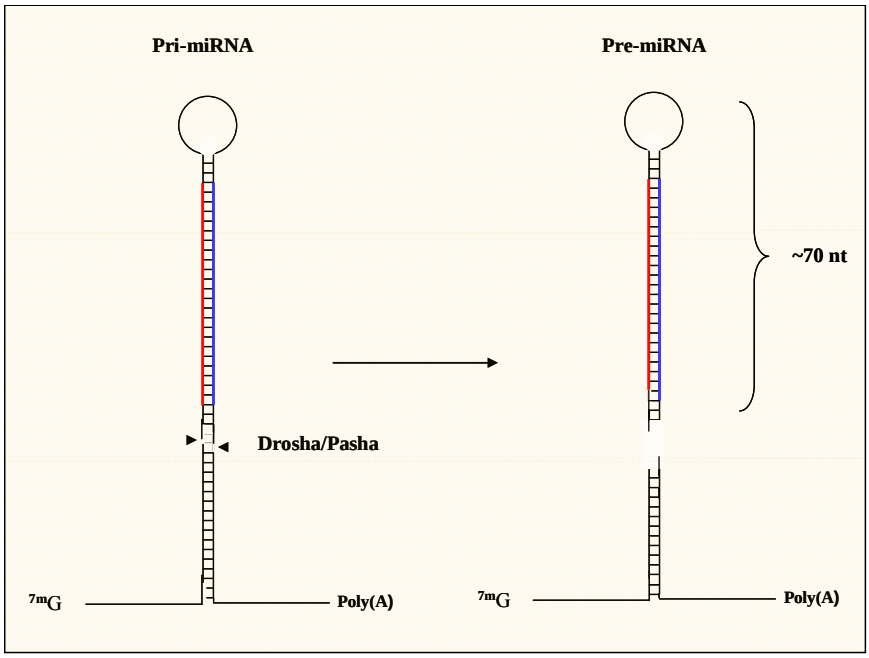

Fig.1.0: Synthesis of pre-microRNA. Schematic representation of the role of Drosha/Dcgr8/Pasha in the production of pre-miRNA from the pri-miRNA precursor molecule.

1.2.3 From pre-miRNA to mature microRNAs

Following its production, the pre-miRNA is bound by the nuclear transport protein Exportin 5 (Bohnsack et al. 2004; Zeng et al. 2004) in a non-sequence specific manner and exported into the cytoplasm via a Ran-GTP gradient (Gwizdek et al. 2003; Bohnsack et al. 2004). Following the relocation of the pre-miRNA molecule into the cytoplasm, GTP is hydrolyzed to GDP, the pre-miRNA released and exportin 5 shuttled back into the nucleus (Gwizdek et al. 2003; Bohnsack et al. 2004). The pre-miRNA is then subjected to another shortening process by Dicer, a class III RNAse III, in the cytoplasm (Bernstein et al. 2001; Macrae et al. 2006; MacRae et al. 2007). Dicer binds double stranded pre-miRNA via its helicase and PAZ domain (Cerutti et al. 2000; Zhang

et al. 2004; MacRae et al. 2007) which derives its name from the homology with three proteins named Piwi, Argo and Zwille (Cerutti et al. 2000) (Figure 1.1).

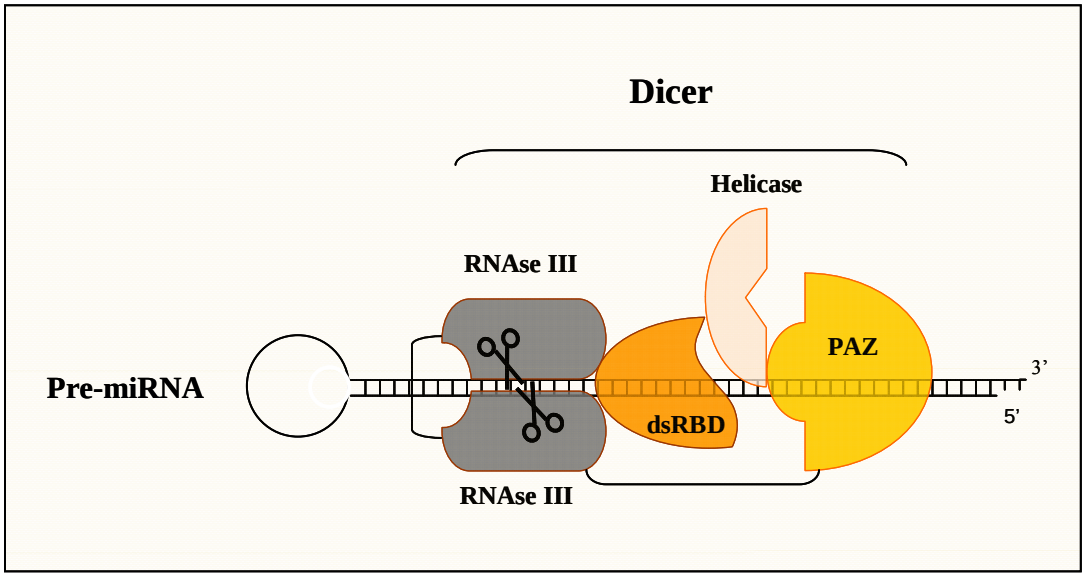

Fig. 1.1: Domains of Dicer. Schematic representation of Dicer. Refer to the text for the function of the various domains. dsRBD=double stranded RNA Binding Domain.

The catalytic domain of Dicer cuts the dsRNA in a non-sequence dependent manner (Basyuk et al. 2003; Lee et al. 2003) at a definite distance from its PAZ domain (Bernstein et al. 2001; Lee et al. 2002; Zhang et al. 2004) producing 22-23 nucleotide long dsRNA linear molecules with two or three nucleotide overhang at the 3' end (Zhang et al. 2004; MacRae et al. 2007). By moving along the pre-miRNA precursor, Dicer produces 22-23 nucleotide long RNA molecules until all the molecule has been processed (Figure 1.2) (Zhang et al. 2004).

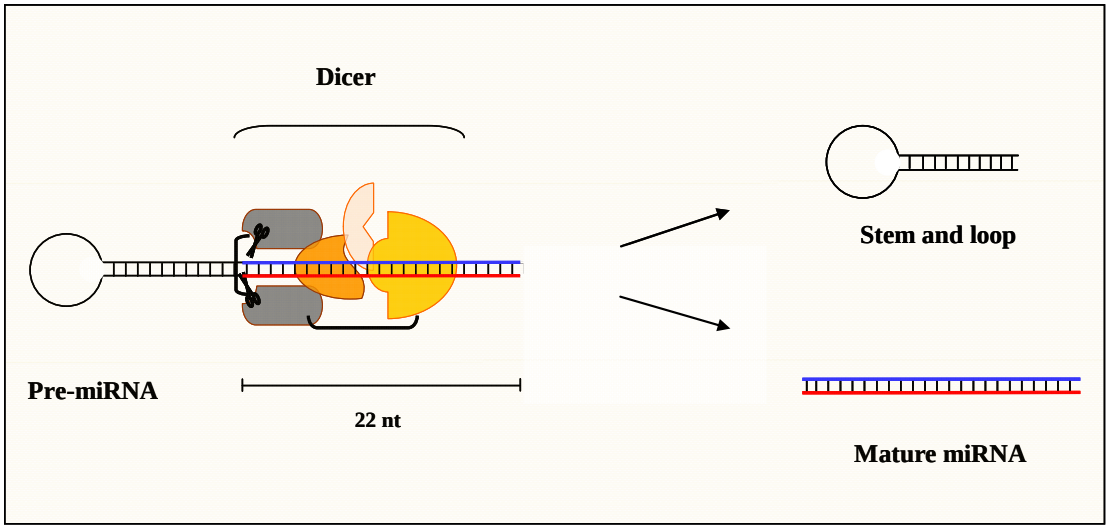

Fig. 1.2: Dicer activity on pre-miRNA. The activity of Dicer on the pre-miRNA leads to the production of a mature 22 nucleotide long double stranded microRNAs. One of the two strands, the "passenger strand" (blue in this figure) is degraded, while the other (red) is the "guide strand" and is incorporated in the RISC where it confers specificity to the silencing complex.

1.2.4 RISC

Of the two RNA strands, one, the guide strand, is incorporated into a multiprotein complex called RISC (RNA Induced Silencing Complex) (Bernstein et al. 2001; Lee et al. 2003; Bartel 2004; Gregory et al. 2005) while the other, the passenger strand, is degraded. Although not yet fully understood, the mechanism that regulates which strand is chosen to become the 'guide' and which the 'passenger', lies in the thermodynamic differences in the 5' ends of each strand (Tomari et al. 2004). The composition of RISC is still being investigated but several proteins have by now been identified (Song et al. 2003; Liu et al. 2004; Forstemann et al. 2005; Gregory et al. 2005; Leuschner et al. 2005). The core component of RISC is made of four Argonaute proteins in mammals

(Ago1-4) and two in Drosophila (Ago1-2). These proteins have conserved PAZ domain (which, as it has been previously described in this thesis, is also present in Dicer) and a PIWI domain which is homologous to RNAseH (Parker et al. 2004; Parker et al. 2005). Other proteins composing RISC include a small dsRBP (Double stranded RNA binding proteins) such as R2D2 (Robb et al. 2007; Kalidas et al. 2008), a ribonuclease termed C3PO which releasesd RISC from the degradation product of the passenger microRNA strand (Liu et al. 2009), proteins with RNA-helicase function (Robb et al. 2007) and the fragile-X mental retardation protein (Jin et al. 2004; Didiot et al. 2009), whose function is still unknown. The microRNA guide strand released by Dicer and incorporated by RISC mediates the specific binding to an mRNA target.

1.2.5 MicroRNA-mediated mRNA degradation

MicroRNAs generally exert their function by binding specific complementary sequences in the 3'UTR of their target mRNA (Rajewsky et al. 2004; Bagga et al. 2005), but it has been recently discovered that they can also bind the coding sequence of mRNAs (Duursma et al. 2008; Tay et al. 2008). The most important part of the microRNA molecules lies towards their 5' end where a region of 5-7 nucleotides named the "seed" has been found to be essential for their function (Lewis et al. 2005; Saetrom et al. 2007). It is generally accepted (Lewis et al. 2003; Brennecke et al. 2005; Hall 2005; Lewis et al. 2005) that, in case of perfect match between the microRNA and mRNA, the latter is cleaved by the RNAseH activity of Ago proteins near the 11^{th} nucleotide from the 5' end of the miRNA (figure 1.3) (Lewis et al. 2003; Brennecke et al. 2005; Hall 2005;

Lewis et al. 2005). This is followed by the degradation of the truncated mRNA molecules.

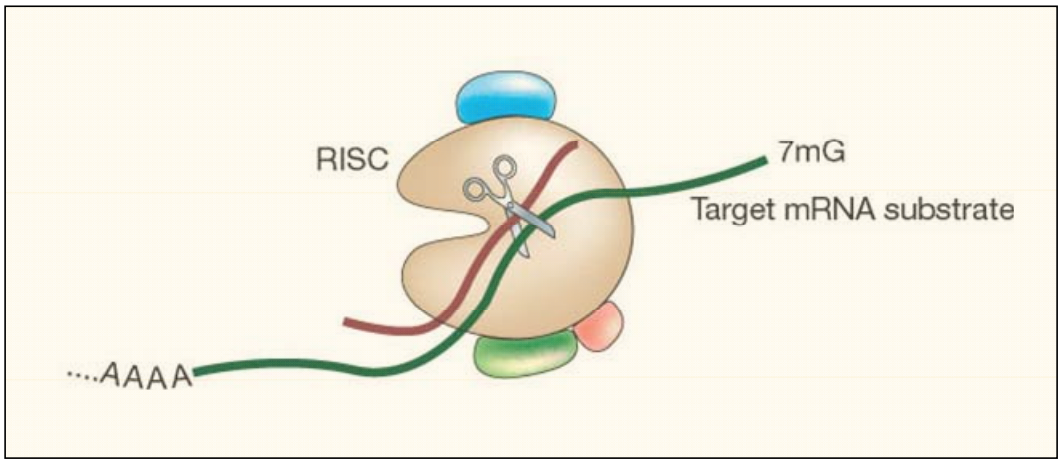

Fig.1.3: Degradation of a target mRNA mediated by a specific microRNA. The target mRNA (green molecule) is bound by specific microRNA (red molecule) incorporated in the RISC and cut.
(Hannon et al. 2004).

1.2.6 P-bodies

In case of mismatch between the microRNAs and its target sequence, the mRNA is not degraded and it becomes part of a multiprotein complex known as P-body (Andrei et al. 2005; Liu et al. 2005; Rehwinkel et al. 2005; Parker et al. 2007; Takimoto et al. 2009). This is composed by mRNA molecules and proteins with different functions such as proteins that constitute the decapping machinery which is involved in the removal of the methyl-guanosine found at the 5' of mRNAs (Dcap-1, Dcap-2, Edc3-p) (van Dijk et al. 2002; Sakuno et al. 2004), poly(A)-tail removal proteins such as the Ccr4p/Pop2p/Not complex (Parker et al. 2007), RNA binding proteins, translation initiation factors such as

eIF4E-T etc (Andrei et al. 2005; Wilczynska et al. 2005), and exonucleases such as (Xrn1p) (Ingelfinger et al. 2002). Messenger RNAs can also exit their association with P-bodies and re-enter translation (Brengues et al. 2005). For example, it has been shown that yeast cells in a stationary growth phase have increased numbers of P-bodies with associated mRNAs (Brengues et al. 2005). These molecules can then re-enter translation following growth stimuli (Brengues et al. 2005).

Messenger RNAs can also be incorporated in P-bodies by a quality control process that degrades mRNAs with aberrant translation termination codons, a phenomenon called Nonsense-Mediated Decay (NMD) (van Dijk et al. 2002; Amrani et al. 2004). MicroRNAs with shortened 3'UTR or the stalling of a ribosome on a mRNA molecule during its translation are other mechanism which can lead these molecules to be incorporated into a P-body (Amrani et al. 2004; Maquat 2004; Inada et al. 2005).

As schematically represented in the figure below (figure 1.4), the equilibrium between mRNAs being translated and sequestered by P-bodies seems to be based on the competition between the assembly of the translation complex and the assembly of the P-body (figure 1.4) (Parker et al. 2007) which is in turn affected by the mechanisms regulating intracellular mRNA fate described above.

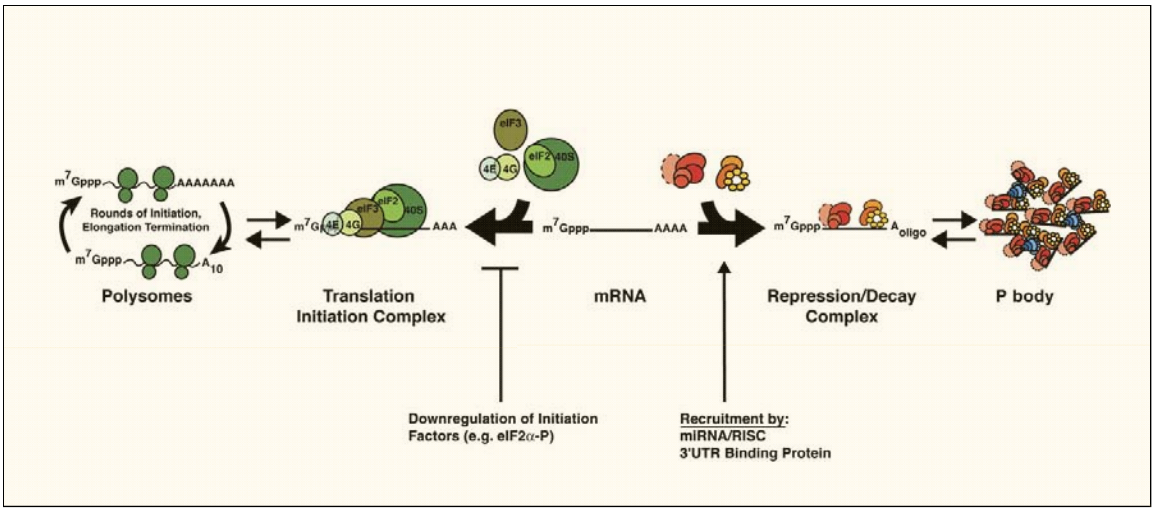

Fig. 1.4: P-Bodies and their role in mRNA translation. An mRNA molecule can be shuttled from its inactive and hibernated state whilst contained in P-bodies (right of the figure) to being actively translated (left of the figure).
(Parker et al. 2007).

1.2.7 MicroRNA production and activity: a summary

The figure below (figure 1.5) summarizes the entire process which leads to the production of mature microRNAs form their synthesis as pri-miRNA, their initial shortening into pre-miRNA by Drosha, their active translocation into the cytoplasm, maturation to 22 nucleotide long microRNAs by Dicer, ending in their incorporation in RISC and in either the degradation of a target mRNA or the incorporation of the latter in P-bodies.

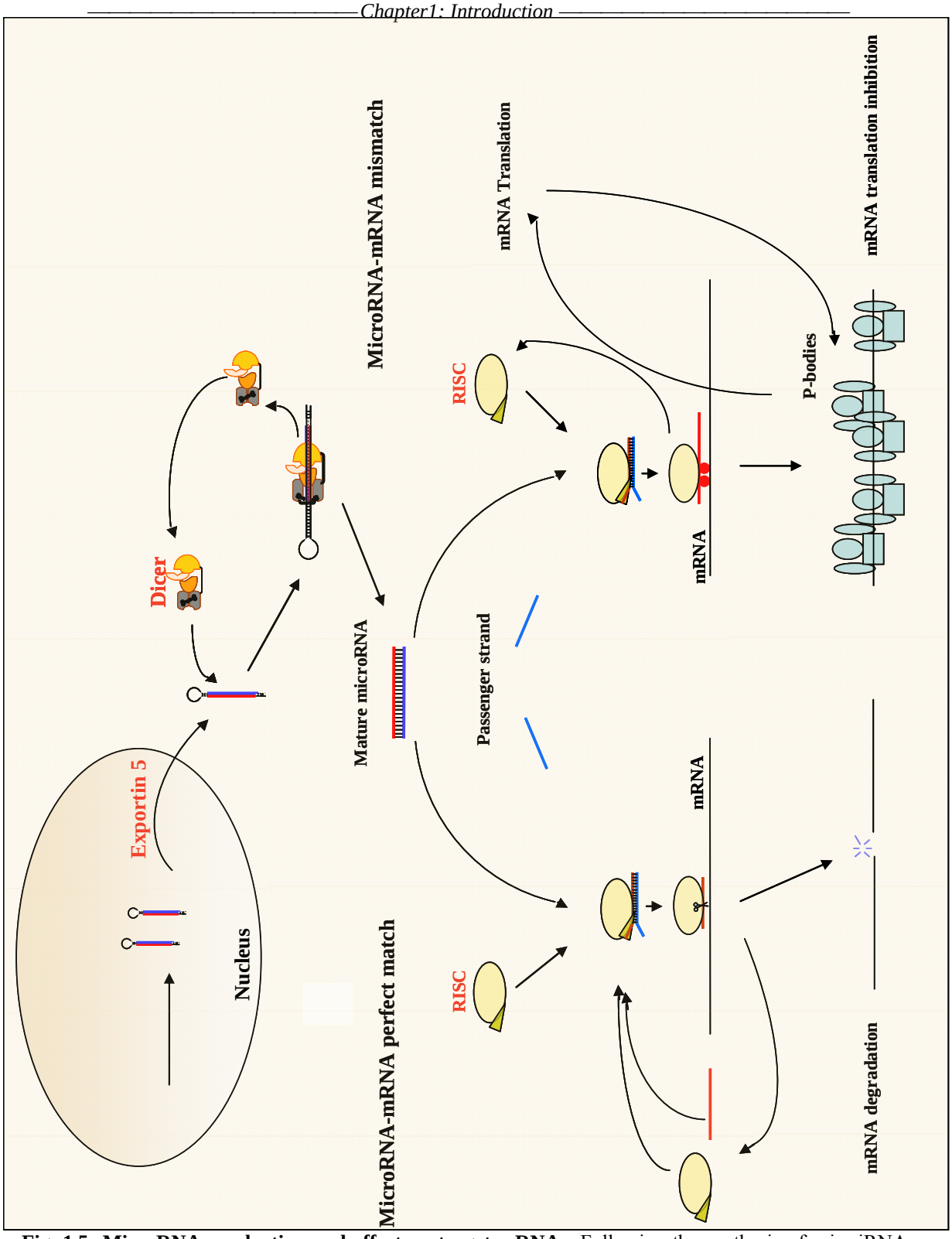

Fig. 1.5: MicroRNA production and effect on target mRNAs. Following the synthesis of pri-miRNA, their initial processing by Drosha in the nucleus and Dicer in the cytoplasm, a mature microRNA binds its target mRNA. In case of perfect match, the mRNA is degraded (left side) while in case of mismatches between the microRNA and its target mRNA, the latter is incorporated into a multiprotein complex (P-bodies) and becomes translationally silent. MRNAs can be released from P-bodies and become translationally active.

It has been estimated that 10-30% of mRNAs are targeted by microRNAs (Brennecke et al. 2003; Brennecke et al. 2005) and that every microRNA is on average potentially able to modulate the expression of circa 2000 different mRNAs (Brennecke et al. 2005; Lewis et al. 2005). More than 5000 unique microRNAs have been identified up to now and classified from more than 50 species (Griffiths-Jones et al. 2008). A recent analysis (Chiang et al. 2010) has confirmed the expression of at least 500 different microRNAs in mice but their number is constantly growing. Human microRNAs alone have been predicted to target circa 30% of human genes and it appears that each microRNA can target circa 200 transcripts (Krek et al. 2005; Lewis et al. 2005; Xie et al. 2005).

Intriguingly, a combination of available experimental data and in-silico analysis regarding microRNA and mRNA expression profiles in different human tissues is allowing the definition of cell-type-specific miRNA "signatures" (Sood et al. 2006). This implies that for any given microRNA expression profile, it would be theoretically possible to infer which mRNAs are possibly expressed in that same tissue (Sood et al. 2006). Although more experimental data is needed in order to draw an accurate correlation between microRNAs and mRNAs expression in any given tissues and whether this has any physiological role, microRNAs can nonetheless be classified according to the tissue in which they are expressed or are expressed in higher proportion compared to other tissues. An example, concerning mammal microRNAs is summarized in the table below (table 1.1).

Expression pattern	microRNA
Enriched in brain	*miR-12a, miR-125b, miR-128, miR-132, miR-139, miR-7, miR-9, miR-124a, miR-124b, miR-135, miR-153, miR-149, miR-183, miR-190, miR-219*
Enriched in lung	*miR-18, miR-19a, miR-24, miR-32, miR-130, miR-213, miR-20, miR-141, miR-193, miR-200b*
Enriched in spleen	*miR-99a, miR-127, miR-142a, miR-142s, miR-151, miR-189, miR-212*
Enriched in liver	*miR-122a, miR-152, miR-194, miR-199, miR-215*
Enriched in heart	*miR-1b, miR-1d, miR-133, miR-206, miR-208, miR-143*
Enriched in kidney	*miR-30b, miR-30c, miR-18, miR-20, miR-24, miR-32, miR-141, miR-193, miR-200b*
Enriched in haematopoetic tissues	*miR-181, miR-223, miR-142*
Ubiquitously expressed	*miR-16, miR-26a, miR-27a, miR-143a, miR-21, let-7a, miR-7b, miR-30b, miR-30c*

Table 1.1: **Example of the expression profile of microRNAs in different human tissues**.
(He et al. 2004)

1.3 General role of microRNAs

Besides being an extremely powerful tool in the hands of scientists whishing to knock-down the expression of specific genes, microRNAs actually play an essential role in several aspects of cellular physio-pathology.

In order to understand the overall role played by the RNAi pathway during development, Dicer and Dgcr8 have been experimentally knocked-out in mice (Bernstein et al. 2003; Wang et al. 2007; Ma et al. 2010; Suh et al. 2010). As further discussed in the following paragraph (1.4), the genetic ablation of Dicer in mice leads to embryonic death

(Bernstein et al. 2003) and it is essential for mice oocyte maturation (Murchison et al. 2007) whilst the elimination of Dgcr8, although not lethal, is associated to mice embryonic arrested development (Wang et al. 2007) but has no effect on oocyte maturation (Suh et al. 2010). Furthermore, while the deletion of Dicer is associated with mRNAs mis-regulation (Suh et al. 2010), Dgcr8 knock out and wild type mRNA profiles are virtually identical (Suh et al. 2010). Due to the essential role played by Drosha in the biosynthesis of microRNAs (Lee et al. 2003; Denli et al. 2004; Gregory et al. 2004; Han et al. 2004; Landthaler et al. 2004), these data suggest that endogenous siRNAs, rather than microRNAs, are at the basis of the Dicer knock-out phenotype (Suh et al. 2010). Intriguingly, miRNA-mediated mRNA degradation is reduced during oocyte development in mice, a phenomenon which might be linked to increased mRNA stability in growing oocytes (Ma et al. 2010).

In regard of other possible role played by Dicer in cellular physiology it has also to be mentioned its functions in the formation of heterochromatin (Pal-Bhadra et al. 2004) and centromeres (White et al. 2004) via mechanisms which are still being investigated (Halic et al. 2010).

Several groups have been analyzing the role of microRNAs in different aspects of development and disease and some of these will be discussed in the following paragraphs.

1.3.1 Stem cells

The existence of cells with the capability of differentiating in many different cell types was firstly demonstrated following the injection of hematopoietic tissues in

irradiated mice (Becker et al. 1963). The implanted cells gave rise to macroscopic colonies which started differentiating along the erythrocytic, granulocytic and megacaryocitc lines (Becker et al. 1963). The term 'stem' cell derives from "meristem" which means "to divide" in ancient greek and was first coined by the botanist Karl Wilhelm von Nägeli (Nägeli 1858) and describes cells with the potential of differentiating in several cell types. The term stem cell is applied to both embryonic stem cells and adult stem cells (Haskill et al. 1970; Micklem et al. 1972; Stent 1985). The former are cells of the blastocyst which will give rise to all the different specialized adult cell types and organs that compose an adult organism (Evans et al. 1981), while the latter are found in several organs and tissues and provide a source of cells which replace the ones lost through normal wear and tear (Ito et al.; Harrison 1979; Simon et al. 1995; Bazan et al. 2004; Simon-Assmann et al. 2007).

As mentioned above, the experimental deletion of Dicer in mice, leads to embryonic death which seems to be due to the depletion of stem cells (Bernstein et al. 2003; Wang et al. 2007) while Dgcr8 knock-out mice embryos have an arrested development, due to the inability to silence embryonic stem cells self-renewal capability when exposed to differentiation stimuli (Wang et al. 2007; Melton et al. 2010). The latter phenotype can be rescued by the re-introdcution of Dgcr8 (Wang et al. 2007) or by the introduction of stem-cells specific microRNAs belonging to the mir-290 family (Wang et al. 2008). Interestingly, the Lin-7 microRNA family has an opposite role to the mir-290 cluster in embryonic stem cells in mice (Melton et al. 2010) whereby let-7 inhibits and mir-290 indirectly activates a number of genes involved in self-renewal (Melton et al. 2010). Intriguingly, the mir-290 cluster is amongst the most abundantly expressed

miRNA in mouse embryonic stem cells (Judson et al. 2009) and has a similar function to the mir-302 cluster in human embryonic stem cells (Lin et al. 2008; Wang et al. 2008).

Intriguingly Dicer has also been found to play an indirect role in the methylation state of Xist promoter (Brown et al. 1991; Nesterova et al. 2008) in embryonic stem cells by regulating the levels of methyltransferase in these cells (Nesterova et al. 2008).

1.3.2 Cancer

As mentioned in section 1.2, *Lin-4* and *Lin-7* were the first microRNAs to be identified and they are both involved in the developmental timing and overall cell growth of the nematode C. *elegans* (Lee et al. 1993; Reinhart et al. 2000). Because of these features, several studies (Calin et al. 2006; Calin et al. 2006; Gaur et al. 2007) have investigated the potential role played by microRNAs in cell-cycle and cell-growth impairment which are often disrupted in cancer and have evidenced that microRNAs profiles are often very different between normal tissues and their cancerous counterparts (Calin et al. 2006; Calin et al. 2006; Gaur et al. 2007). Furthermore, it has been observed that several genes encoding for microRNAs are located in known fragile chromosomal regions often associated with different forms of cancer (Calin et al. 2004).

Both the up-regulation and down-regulation of microRNAs have been found associated with cancer. For instance, chronic lymphocytic leukemia (CLL) is often associated with a deletion on chromosome 13 in humans (Pekarsky et al. 2005). Although this region does not contain any evident gene(s) causally involved in this form of CLL, it was found that it encodes for two miRNAs, mir-15a and mir-16a, whose expression is

frequently down-regulated in patients with B cell chronic lymphocytic leukemia (Calin et al. 2004; Pekarsky et al. 2005). Further examples concern the reduction of miR-143 and miR-145 expression which is directly associated to colorectal neoplasia in humans (Michael et al. 2003) or the expression levels of mir-22 which are often reduced in some human breast cancers (Xiong et al. 2010). Conversely, it has been described that mir-155 is up-regulated 100-fold in patients with Burkitt's lymphoma (Metzler et al. 2004). Increased levels of the mir-17-92 cluster are found instead in patients with B cell lymphoma (He et al. 2005). Interestingly, the artificial manipulation of the expression levels of this microRNA cluster in mice is associated to increased incidence of lymphomas (He et al. 2005). Because if its pro-tumorigenic capacity, the mir-17-92 cluster has been named oncomir-1, the first microRNA with oncogenic properties (He et al. 2005). Another oncomir, mir-21, has been shown to have a causative role in the development and progression of hepatocellular carcinomas by regulating cell proliferation, apoptosis and tumor invasiveness (Liu C 2010).

Beside their role as oncogenes, microRNAs can also have a tumor-suppressor activity. The reduction of let-7 microRNA levels, for instance, is associated to an increase of RAS protein levels in several forms of lung cancer (Johnson et al. 2005) and the artificial over-expression of this microRNA induces a down-regulation of RAS levels which results in reduced cellular growth (Johnson et al. 2005). Another example concerns the role of mir-519 in the suppression of tumor growth (Abdelmohsen et al. 2010). This microRNA targets the mRNA encoding for HuR (Calaluce et al. 2010), an RNA binding protein which has been shown to bind AU-rich elements (ARE) of proto-oncogenes, thus protecting them from degradation (Calaluce et al. 2010). Furthermore, increased levels of

the mir-103/107 are found in breast cancer with metastasis and unfavourable outcome (Martello 2010). The experimental inhibition of this microRNA reduces metastatic capability of malignant cells. Intriguingly, the mir-103/107 miRNA targets Dicer mRNA and this leads to a global reduction of miRNA biosynthesis (Martello 2010).

The recent identification of microRNAs in human plasma (Mitchell et al. 2008), offers also an intriguing diagnostic possibilities regarding the identification of microRNA signatures associated to specific cancers (Mitchell et al. 2008) as exemplified, for instance, by the detection of mir-141 in the serum of patients with prostate cancer (Mitchell et al. 2008).

1.3.3 Neuronal cells

The role played by microRNAs in the development of the nervous system was initially established in zebrafish mutants in which the RNAse III and RNA-binding domain of Dicer had been disrupted (Giraldez et al. 2005). This resulted in abnormal somitogenesis, heart development and brain formation (Giraldez et al. 2005). The specific role played by microRNAs in brain development in zebrafish Dicer mutants, was established by the rescue of the brain defects following the injection of mir-430 (Giraldez et al. 2005; Giraldez et al. 2006). A microarray approach and in vivo validation has shown that mir-430 can target several hundred mRNAs (Giraldez et al. 2006) and that it is involved in the elimination of maternal mRNAs during embryogenesis (Giraldez et al. 2006).

The first neuronal disease to be identified as being associated with the microRNAs pathway is the Fragile X syndrome (FXS), an inherited disease causing learning disabilities and delayed speech and language development (Caudy et al. 2002; Jin et al. 2004). The disease is linked to the *Fmr1* gene which, as mentioned before, encodes for a protein (Caudy et al. 2002) part of RISC, the multiprotein complex involved in the production of microRNAs (Caudy et al. 2002; Song et al. 2003; Liu et al. 2004; Forstemann et al. 2005; Gregory et al. 2005; Leuschner et al. 2005). Other examples include mir-106 and mir-520c which, for instance, can modulate the expression of the amyloid precursor protein (APP), implicated in Alzheimer's disease (AD) (Patel et al. 2008). Another neurodegenerative disease, Parkinson's disease (PD) (Parkinson 1817), has been found to be associated in some patients to a mutation in the 3'UTR of the fibroblast growth factor 20 (*Fgf20*) (Wang et al. 2008) which might impair the binding of mir-433 (Wang et al. 2008). *Fgf20* activates the expression of α-synuclein (Forloni et al. 2000) which is associated to a form of Parkinson's disease (Forloni et al. 2000; Wang et al. 2008). The mutations on the 3'UTR of Fgf-20 mRNA thus results in elevated levels of *Fgf20* which induces an increase of the levels of α-synuclein (Wang et al. 2008) .

The identification of Dicer, mRNAs and ribosomes at synaptic terminals (Steward et al. 1982; Lugli et al. 2005; Schuman et al. 2006), has triggered an interest in understanding the mechanisms regulating translation at distant locations from the nucleus (Steward et al. 1982; Lugli et al. 2005; Schratt et al. 2006). It has been established, for instance, that mir-134 plays a direct role in synaptic plasticity by regulating dendritic spine size via the down-regulation of Limk1 mRNA, a protein kinase involved in synaptic development (Schratt et al. 2006). So far, more than 80 different microRNAs

have been found at synaptic terminals (Kim et al. 2004) and most importantly Dicer is enriched at postsynaptic densities suggesting that microRNAs might be produced *in situ* (Lugli et al. 2005). Furthermore, microRNAs have been found to be expressed in dendrites of explanted rat dorsal root ganglion neurons stimulated with trophic factors such as NGF or NT3 (Hengst et al. 2006).

It has also been recently discovered that mRNAs, microRNAs and proteins can be transferred across synapses inside exosomes (Faure et al. 2006; Gibbings et al. 2009), small lipid vesicles derived from multivesicular bodies (MVBs) of the endocytic pathway (Faure et al. 2006). This opens a new possibility of inter-cellular communication and post-transcriptional gene regulation

1.3.4 Cardiogenensis

Cardiovascular development provides another example of the direct role played by microRNAs in organogenesis. For instance, mir-1/2, mir-133 and mir-206 are expressed and have a role in cardiac and skeletal muscles (Kwon et al. 2005; Zhao et al. 2005; Chen et al. 2006). In particular, the mir-1/2 cluster is expressed from E8.5 in the mouse and increases throughout development (Zhao et al. 2007). The experimental deletion of this cluster in mice, results in ventricular septal heart defects and occasional heart oedema (Zhao et al. 2007) which is likely to be caused by an impaired regulation of *Hand2*, an essential basic helix-loop-helix transcription factor involved in the growth of ventricular myocyte (Zhao et al. 2005; Zhao et al. 2007). A precise dosage of *Hand2* is required for normal cardiomyocyte development and morphogenesis (Srivastava et al.

1995; Yelon et al. 2000; McFadden et al. 2005) and the control of its expression levels by mir-1/2 is thus essential for normal cardiac development (Zhao et al. 2007). Intriguingly, mir-1/2 plays an antagonistic role to mir-133 (Chen et al. 2006). Whereas mir-1/2 promotes myogenesis by targeting histone deacetylase 4 mRNA, miR-133 instead enhances myoblast proliferation by repressing serum response factor (SRF) (Chen et al. 2006).

Beside its role in cardiogenesis, microRNAs have been shown to be involved in development of the vascular system (Fish et al. 2008; Suarez et al. 2008; Nicoli et al. 2010). The lack of mir-126, for example, leads to collapsed blood vessels and cranial haemorrhages in zebrafish (Fish et al. 2008). This microRNA is the most abundantly expressed microRNA in zebrafish endothelial cells and exerts its activity by targeting Spred1 and PIK3R2 mRNAs which are both involved in the response of endothelial cells to vascular endothelial growth factor (VEGF) (Fish et al. 2008). In contrast, miR-92a represses the growth of new blood vessels by targeting mRNAs encoding for pro-angiogenic proteins such as the integrin subunit alpha5. (Bonauer et al. 2009). These results have suggested the possibility of suppressing or inducing the expression of specific microRNAs as a way to control neo-angiogenesis in tumours or viceversa to stimulate blood vessels growth in ischemic tissues (Dews et al. 2006; Hua et al. 2006; Poliseno et al. 2006; Kuehbacher et al. 2007; Bonauer et al. 2009).

1.3.5 MicroRNA and transcription factors

MicroRNAs have also been found to be involved in the regulation of mRNAs encoding for many kinds of proteins including transcription factors (Wan et al.; Hobert 2004; Szulwach KE 2010). For instance, the expression of the E2F3a transcription factor, which along with other members of the E2F family regulates cell proliferation and apoptosis (Paulson et al. 2006; Chong et al. 2009), is tightly regulated by mir-128 (Zhang et al. 2009). The down-regulation of this microRNA leads to increased levels of E2F3a which in turn causes oncogenic transformation of glia cells (Zhang et al. 2009).

An even more interesting example concerns the regulation of the *Hox* network by microRNAs. *Hox* genes are a group of highly related and conserved genes which regulate the formation of the embryonic anterior-posterior axis (Colberg-Poley et al. 1985; Manley et al. 1985; Fienberg et al. 1987; Holland et al. 1988; Schughart et al. 1988; Krumlauf 1994; Matsuo et al. 1995). Mammals have four HOX clusters (A to D) which contain 39 genes (Krumlauf 1994). They encode for transcription factors which share a conserved domain named homeobox (Schneuwly et al. 1986; Gehring 1987; Kessel et al. 1987). Their expression is time and space regulated, i.e. each *hox* gene is activated after the one preceding it in the same cluster (Deschamps et al. 1993; Tschopp et al. 2009). Interestingly, the mir-196 family, which is encoded by genes interspersed within the *HoxA, B and C* clusters (Yekta et al. 2004), targets the 3'UTR of Hox genes representing each cluster: HoxB8, HoxC8, HoxD8 and HoxA7 (Mansfield et al. 2004; Yekta et al. 2004). Members of the mir-10 family, instead, are expressed from within Hox-4 paralogues and target HoxB1a and HoxB3a (Mansfield et al. 2004; Woltering et al. 2008).

The Brn-3 transcription factors, composed of Brn-3a, Brn-3b and Brn-3c, share some characteristics with the *Hox* family, mainly a DNA binding homeodomain in their C-terminus (Gruber et al. 1997; Morris et al. 1997) and are involved in several aspects of neuronal survival and differentiation (Collum et al. 1992; Gerrero et al. 1993; Turner et al. 1994; Gan et al. 1996; Xiang et al. 1998).

Surprisingly, in contrast with the lack of any conservation in the 3' untranslated region of Brn-3a and Brn-3c, the 3'UTR of Brn-3b is remarkably conserved across different species (Calissano et al. 2007). This suggests that this region might have a role in the regulation of Brn-3b expression in specific cell types. The analysis of this region and its role in the regulation of Brn-3b constitutes the other focus of this thesis but firstly the Brn-3 family of transcription factors will be analysed and their function described.

1.4 POU IV transcription factors

As reported in the previous paragraph, Hox proteins are critical in the anterior-posterior body axis formation (Fienberg et al. 1987; Schughart et al. 1988) and characterized by the presence of a 60 aminoacid conserved homeodomain which binds DNA (Colberg-Poley et al. 1985; Manley et al. 1985; Fienberg et al. 1987; Holland et al. 1988; Schughart et al. 1988; Krumlauf 1994; Matsuo et al. 1995). This domain is characteristic of other families of transcription factors such as the Brn-3a, Brn-3b and Brn-3c proteins (Collum et al. 1992; Gerrero et al. 1993; Ninkina et al. 1993; Xiang et al. 1993; Turner et al. 1994). These are members of the POUIV class of transcription factors and are expressed in different parts of the central and peripheral nervous system (Sturm et

al. 1988; Collum et al. 1992; Gerrero et al. 1993; Turner et al. 1994; Gan et al. 1996; Xiang et al. 1998). Members of the POUIV family, beside a typical homeodomain shared with other homeobox proteins (Sturm et al. 1988), are characterized by the presence of another DNA binding domain (DBD), the POU specific domain, which was initially defined after its identification in the Pit1, Oct-1/2 and Unc-86 transcription factors (Sturm et al. 1988; Sturm et al. 1988) (figure 1.8 and 1.9). As shown in the figure below (figure 1.6), POUIV members are a sub-group within the POU family of transcription factors.

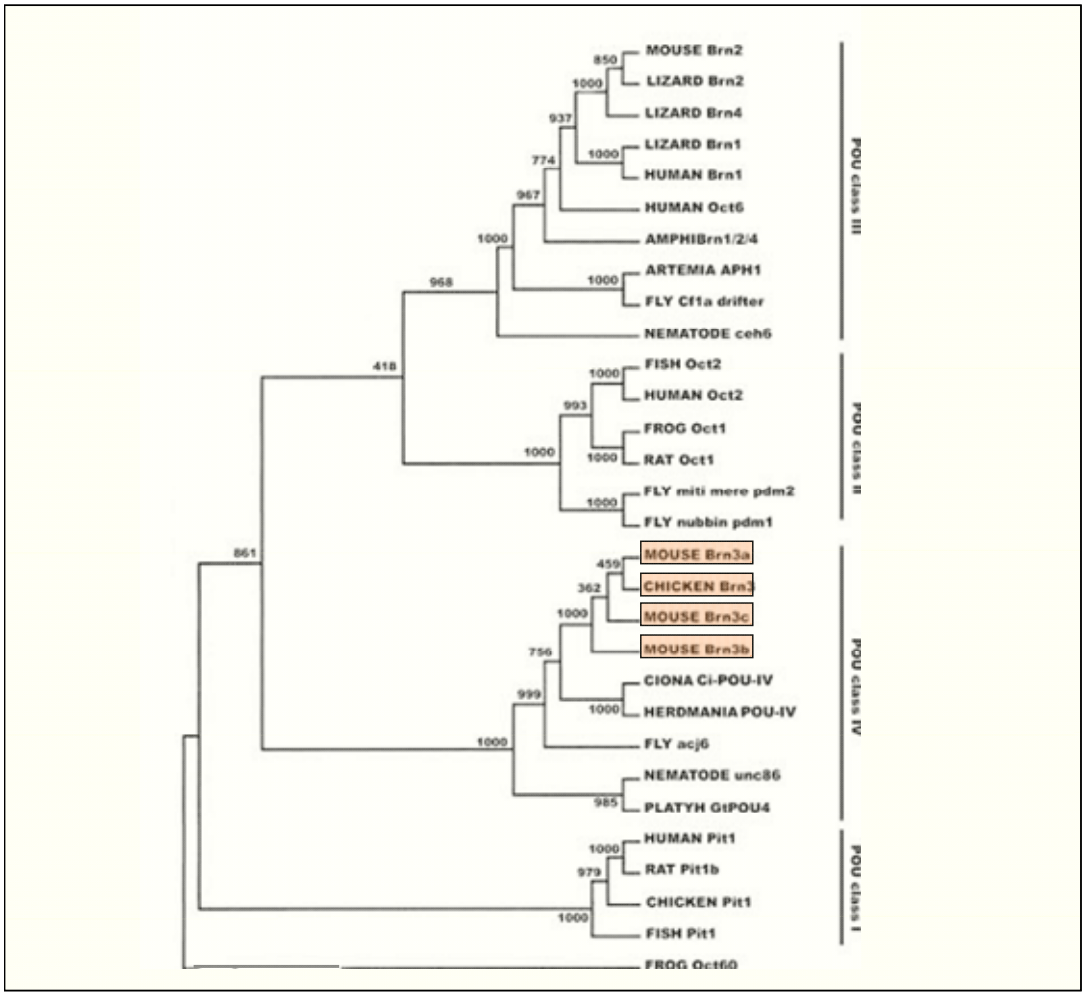

Fig. 1.6: POU family of transcription factors. Evolutionary tree representing the POU family of transcription factors. The mouse and chicken Brn-3a, Brn-3b and Brn-3c POUIV members are highlighted by an orange box. From: (Candiani et al. 2005)

Members of the POUIV class, besides the C-terminal POU domain also share a short conserved region in their N-terminus, the POUIV box (figure 1.7) (Theil et al. 1993). The highest homology between the Brn-3 transcription factors is in their C-terminal DBD while their N-terminus shares a moderate conservation amongst family members (figure 1.8 and 1.9) (Collum et al. 1992; Ring et al. 1993). Due to the focus of this thesis on Brn-3b and Brn-3a TFs and due to the lower degree of homology shared by Brn-3c with the other two members of the POUIV family, Brn-3c will not be analyzed or discussed further in this thesis.

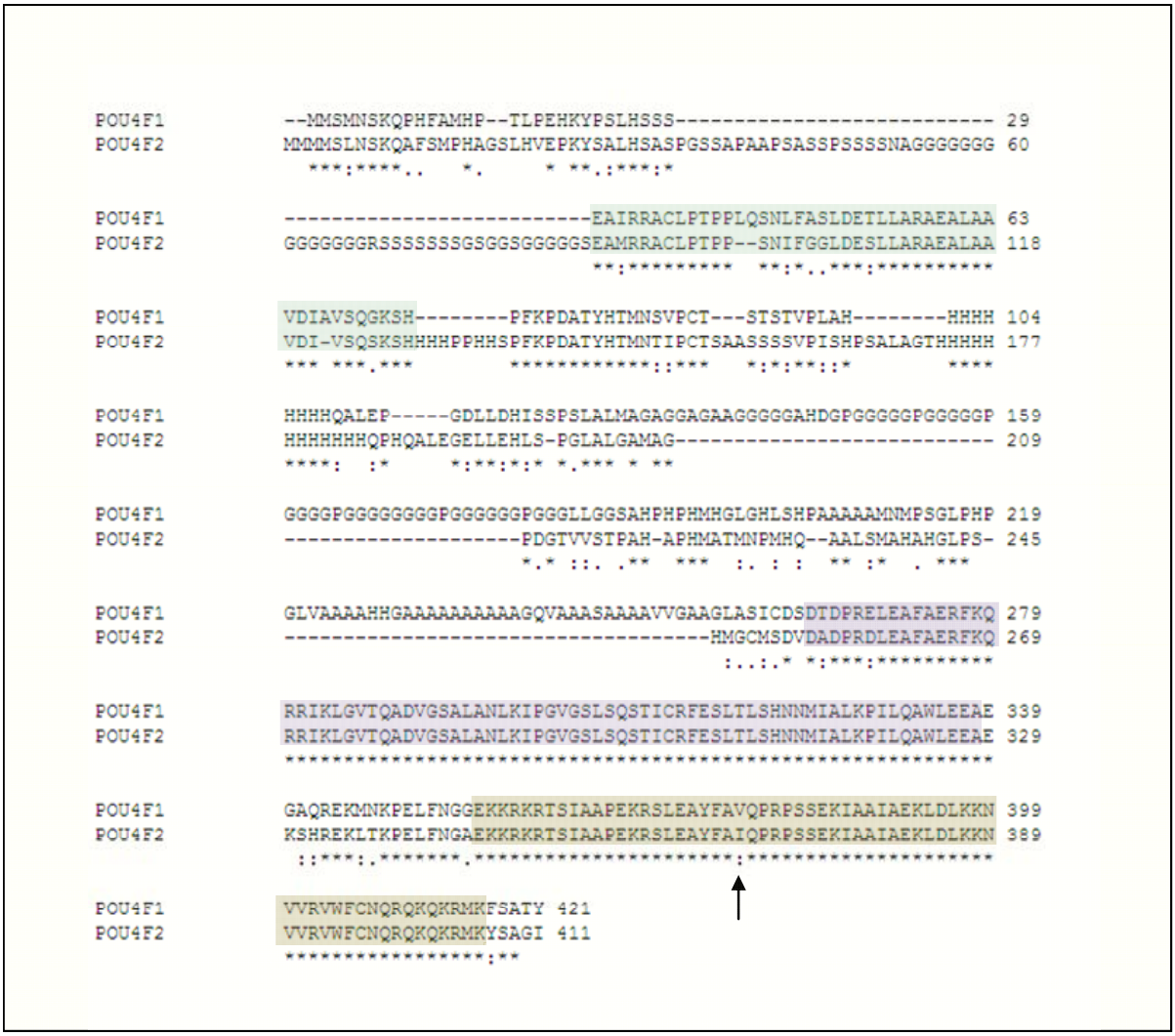

Fig.1.7: Homology of Brn-3a (POU4F1) and Brn-3b (POU4F2) proteins. The green shaded box indicates the POU IV box, the purple the POU domain and the yellow the homeodomain. The arrow indicates the position of the essential wild type Val and Ile (in Brn-3a and Brn-3b respectively) in the homeodomain. The artificial substitution of this aminoacid transforms Brn-3a-Val/Ile into a repressor and Brn-3b-Ile/Val into an activator (Morris et al. 1997).

1.4.1 Structure of POU IV proteins

As shown in figure 1.7 and schematically in figure 1.8, the C-terminal of Brn-3 proteins is composed of an 80 aminoacid long POU domain (which is present only in the members of this class) (Sturm et al. 1988; Collum et al. 1992) and of a 60 aminoacid long homeodomain (which is shared among other homeodomain proteins) (Gruber et al. 1997; Morris et al. 1997). The two domains bind DNA in a synergistic way (Gruber et al. 1997) although the POU domain has a higher affinity for DNA than the homeodomain (Gruber et al. 1997).

The N-terminus activation domain (AD) of Brn-3a, which contains a highly conserved region, the POUIV box (Gerrero et al. 1993), is able to transform fibroblasts (Theil et al. 1993). Intriguingly, this region is conserved and has been shown to play an essential part in the oncogenic activity of c-myc (Niklinski et al. 2000). As discussed in more detail in the following paragraphs, beside its pro-oncogenic role, the AD plays a very important part in the modulation of transcriptional regulation of Brn-3a.

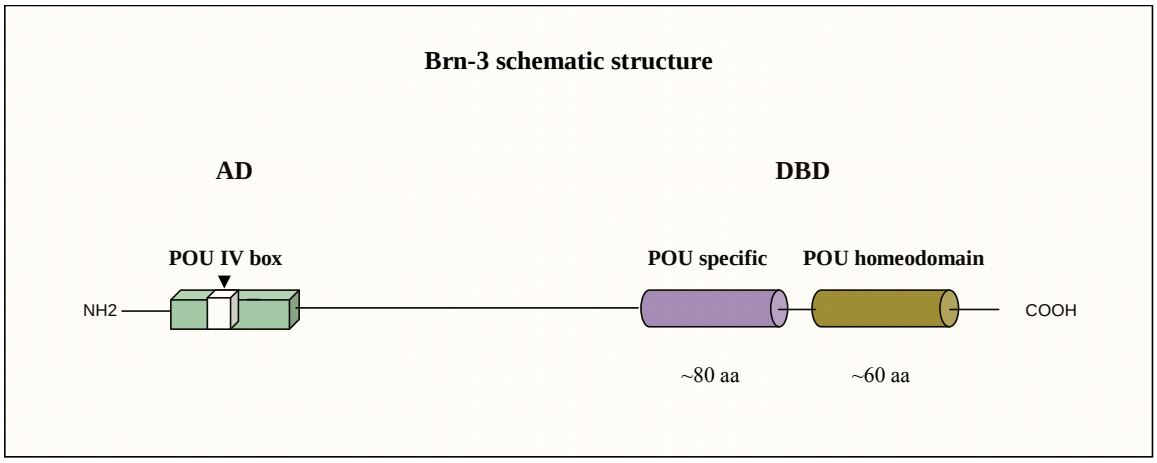

Fig. 1.8: Schematic structure of the Brn-3 proteins. Schematic (not to scale) representation of the Brn-3 protein conserved domains. AD=activation domain, DBD= DNA binding domain. The length, in aminoacids, is indicated below each DBD.

1.4.2 Antagonistic role of Brn-3a and Brn-3b

Both Brn-3a and Brn-3b can be found in two different isoforms, long and short (Theil et al. 1995; Liu et al. 1996; Latchman 1998), which derive from alternatively transcribed mRNAs with the long one having an extra N-terminus domain (figure 1.9) (Liu et al. 1996). It has also been demonstrated that Brn-3a long and short isoform can originate from transcripts produced from two distinct promoters, the short one being in the intron that separates the two exons (Thomas et al. 2004). The long form of Brn-3a (Brn-3aL) is characterized by an extra 84 aminoacids which contain the POU IV box (Liu et al. 1996; Smith et al. 1998) while the long form of Brn-3b (Brn-3bL) has 57 more aminoacids at its N-terminal (Theil et al. 1995). Furthermore, due to an alternative translational start site, the short form of Brn-3b (Brn-3bS) has nine extra amino-terminal residues that are not found in the longer form (Theil et al. 1993; Theil et al. 1995).

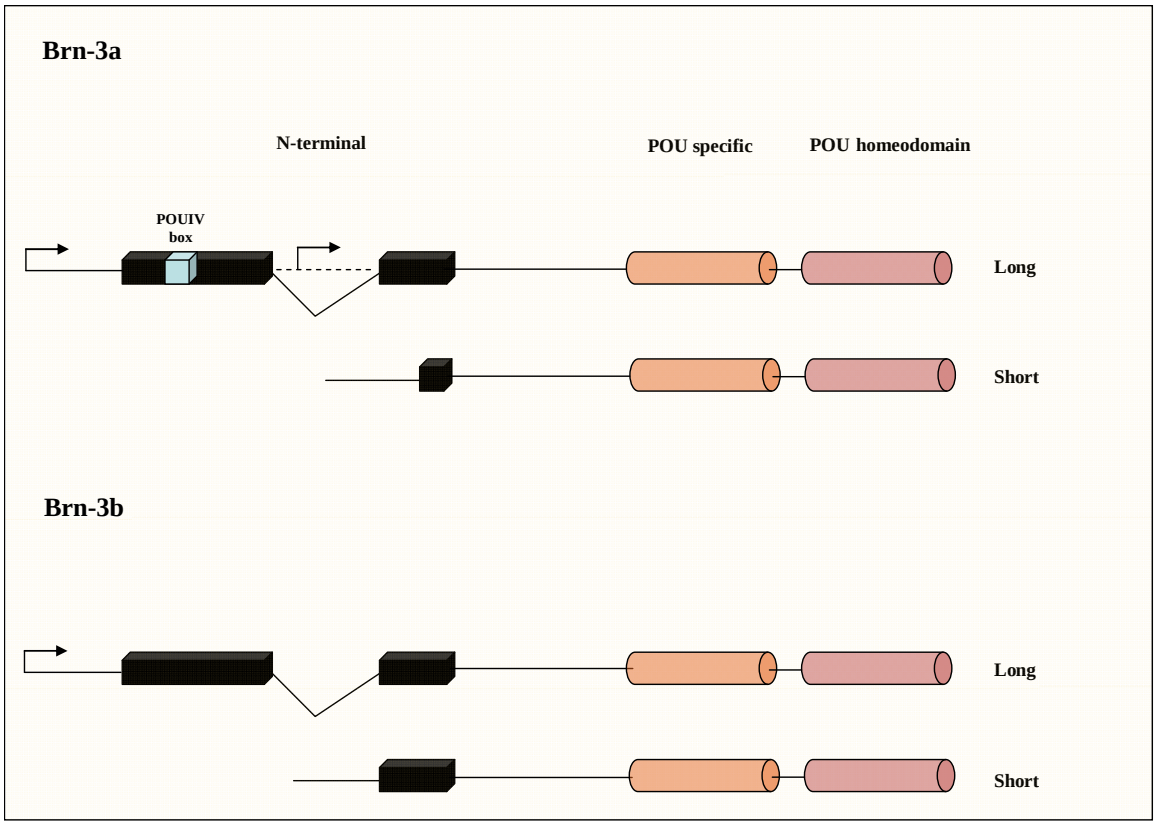

Fig. 1.9: Long and short isoforms of Brn-3 proteins. Schematic representation of the long (L) and short (S) Brn-3a and Brn-3b transcription factors isoforms. Transcription start sites are indicated by arrows. The dotted line in the N-terminal of Brn-3aL indicates a cryptic promoter within its intron which is used for the production of its short isoform.

The two forms differ in their ability to activate the expression of overlapping but different subset of genes. Although both forms of Brn-3a can transactivate neuronal differentiation genes (Lakin et al. 1995; Liu et al. 1996; Latchman 1998; Ma et al. 2003), only the extra 84 amino acids contained at the N-terminus of its long form mediate the transactivation of anti-apoptotic genes such as Bcl-2 and Bcl-x (Lakin et al. 1995; Smith et al. 1997; Smith et al. 1998; Smith et al. 2001).

Several studies have focused on the antagonistic yet complementary role of Brn-3a and Brn-3b in neuronal cells (Morris et al. 1994; Budhram-Mahadeo et al. 1995; Smith et al. 1996). When the ND7 neuroblastoma cell line is induced to differentiate via serum removal, Brn-3b levels decrease while Brn-3a levels increase (Budhram-Mahadeo et al. 1995). This phenomenon is strictly coupled to the differential expression of a subset of genes involved in the cellular routing towards differentiation (Brn-3a) (Lakin et al. 1995; Smith et al. 1998; Smith et al. 1999; Smith et al. 2001; Calissano et al. 2003), or proliferation (Brn-3b) (Budhram-Mahadeo et al. 1998; Dennis et al. 2001; Samady et al. 2004; Lee et al. 2005). Via their POU domain, both transcription factors bind the same octamer target core sequence, AT(A/T)A(T/A)T(A/T)AT with the initial AT generally preceded by a GC dinucleotide (Gruber et al. 1997). The repressive activity of Brn-3b on Brn-3* target promoters lies in the valine at position 378 (figure 1.8) and might be due to the recruitment of a binding partner or to a modification of the POU domain structure that alters its ability to bind DNA (Morris et al. 1997). As mentioned above, Brn-3 proteins can also homo- or heterodimerize (Theil et al. 1995). In Brn-3a and Brn-3b co-transfection experiments in neuronal cells, Brn-3b has a dominant negative effect on the activity of Brn-3a on its target promoters (Morris et al. 1994; Theil et al. 1995).

1.4.3 Promoter activation by Brn-3 proteins

While both long and short forms of Brn-3a and Brn-3b exert their transcriptional activity via their DBD, their N-terminal is essential for the activation of a specific subset

of genes (Latchman 1998; Smith et al. 1998; Calissano et al. 2003; Berwick et al. 2009). So, for instance, the Bcl-2, Bcl-x and α-internexin promoter are activated only by the long form of Brn-3a (Budhram-Mahadeo et al. 1995; Smith et al. 1998; Ensor et al. 2001). This phenomenon can be abolished by the co-expression of the isolated N-terminal of Brn-3a (Smith et al. 1998) suggesting that the activation of the long-form specific promoters require an extra co-factor (Smith et al. 1998). As also mentioned before, the N-terminal of Brn-3a has oncogenic properties as demonstrated by its ability to transform primary fibroblasts (Theil et al. 1993), a phenomenon which is intriguingly abolished upon its co-transfection with Brn-3bS (Theil et al. 1993). This is due to the direct interaction of Brn-3bS and Brn-3aL which leads to the formation of an inactive heterodimer (Theil et al. 1995). Thus Brn-3b, which is also found in breast cancers (Budhram-Mahadeo et al. 1999; Budhram-Mahadeo et al. 2006), can have a cancer suppressive activity when inactivating Brn-3a (Theil et al. 1993).

The Brn-3a and Brn-3b long and short isoforms are expressed in vivo at different ratios which plausibly reflect their requirement at the transcriptional level (Ninkina et al. 1993; Xiang et al. 1993; Liu et al. 1996; Fedtsova et al. 1997; Xiang et al. 1997; Xiang et al. 1997; DeCarvalho et al. 2004; Ichikawa et al. 2005). So, for instance, Brn-3aL is found more expressed in the rat brain and spinal cord (Ninkina et al. 1993; Fedtsova et al. 1997), whereas its short form is more abundant in DRG (Ninkina et al. 1993; Ichikawa et al. 2005). Brn-3bS, instead, is more abundantly expressed in retinal ganglion cells and DRG (Xiang et al. 1993; Xiang et al. 1997; DeCarvalho et al. 2004) and the long form in the spinal cord (Liu et al. 1996).

As reported in the table below (table 1.2), Brn-3a is an overall stronger promoter activator than Brn-3b and in some instances (α-internexin, SNAP-25, herpes simplex promoters) plays an antagonistic role (Budhram-Mahadeo et al. 1995; Budhram-Mahadeo et al. 1995; Lakin et al. 1995; Lillycrop et al. 1995). Furthermore, Brn-3a target genes are generally involved in cellular differentiation, scaffolding and cell survival (Budhram-Mahadeo et al. 1995; Morris et al. 1996; Smith et al. 1996; Smith et al. 1997; Smith et al. 1998; Smith et al. 1999; Sugars et al. 2001; Calissano et al. 2003; Farooqui-Kabir et al. 2004; Berwick et al. 2009) while Brn-3b regulates cell cycle and proliferation (Smith et al. 1996; Budhram-Mahadeo et al. 1998; Budhram-Mahadeo et al. 1999; Samady et al. 2006). For simplification purposes the table below (table 1.2) does not report distinction in the activity exerted by the long and short isoforms of each transcription factor.

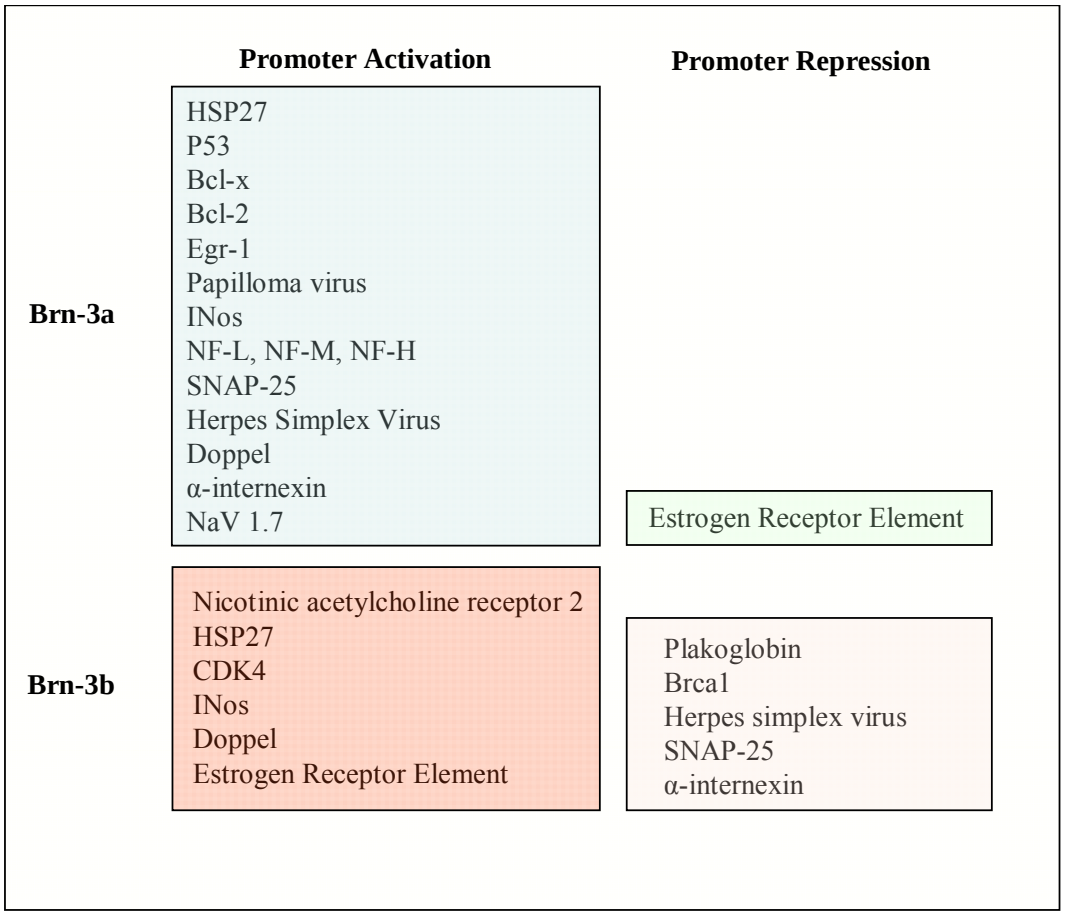

Table 1.2: Promoter activation and repression by Brn-3* transcription factors. Schematic representation of genes activated and repressed by Brn-3a and Brn-3b see (Lillycrop et al. 1995; Milton et al. 1995; Smith et al. 1997; Gay et al. 1998; Smith et al. 1998; Budhram-Mahadeo et al. 1999; Ndisang et al. 1999; Smith et al. 2001; Budhram-Mahadeo et al. 2002; Calissano et al. 2004; Farooqui-Kabir et al. 2004; Samady et al. 2006; Diss et al. 2008).

1.4.4 Modulation of Brn-3a activity

The activity of Brn-3a can be modulated by post-translational modification and interactions with specific co-factors (Theil et al. 1995; Budhram-Mahadeo et al. 1998; Dennis et al. 2002; Thomas et al. 2002; Calissano et al. 2003). It has been previously demonstrated that while the C-terminal of Brn-3a can functionally bind the EWS

transcription factor leading to a reduction in the transactivation of the Bcl-x promoter (Thomas et al. 2002) via its N-terminus, Brn-3a can functionally interact with Rin, a member of the RAS super-family (Calissano et al. 2003). This interaction enhances the ability of Brn-3a to transactivate the egr-1 promoter, a Nerve Growth Factor (NGF) early responsive gene (Calissano et al. 2003). Furthermore, the N-terminus of Brn-3a contains a domain, the POUIV box, which is only found in POU IV proteins (Gerrero et al. 1993) (see figure 1.10). This region, which is highly homologous to a domain of the c-myc family, contains a threonine in position 39 which has been previously shown to be phosphorylated in neuronal cells (Gerrero et al. 1993; Theil et al. 1993; Calissano et al. 2005; Berwick et al. 2009). Un-published data from our laboratory suggest that the phosphorylation of threonine-39 influences the binding between Rin and Brn-3a and might, in turn, affect the transactivation of Brn-3a target promoters. The phosphorylation of Brn-3a on serine 121 and 122, which is promoted by neuronal differentiation stimuli, has the effect of increasing the transactivation of Bcl-2 and Bcl-x, two related anti-apoptotic genes (Calissano et al. 2005).

Furthermore, as mentioned above, one of the key elements at the basis of Brn-3a and Brn-3b different activity is in the valine/isoleucine difference between Brn-3a and Brn-3b at position 378 of their POU homeodomain (figure 1.8, page 48). The change of valine into isoleucine can transform Brn-3a from an activator into a repressor and viceversa Brn-3b from a repressor into an activator (Dawson et al. 1996).

1.4.5 Role of Brn-3 proteins in neuronal survival

All Brn-3* transcription factors play an essential role in the survival and differentiation of a specific subset of neuronal cells (McEvilly et al. 1996; Xiang et al. 1998; Gan et al. 1999). Brn-3a plays an essential role in the survival of sensory neurons of dorsal root ganglia (McEvilly et al. 1996; Huang et al. 1999), while Brn-3b has an analogous role in the retinal ganglion cells of the developing eye (Gan et al. 1999; Mu et al. 2004) and Brn-3c in the ear (Xiang et al. 1993; Xiang et al. 1996; Xiang et al. 1997; Xiang et al. 1998; Clough et al. 2004). Brn-3a knock-out mice die a few days after birth (Xiang et al. 1996), while mice lacking Brn-3b, although viable, exhibit a specific and dramatic loss of up to 70% of retinal ganglion cells (RGC) (Gan et al. 1996; Eng et al. 2001; Wang et al. 2002). Brn-3a is predominantly expressed in sensory neurons of dorsal root ganglia, trigeminal ganglion and in the red nucleus where it plays a pro-survival and anti-apoptotic activity (Collum et al. 1992; Fedtsova et al. 1995; McEvilly et al. 1996). Brn-3b instead is predominantly expressed in developing and adult RGCs (Theil et al. 1993; Xiang et al. 1993). In mice, Brn-3b can normally be detected as early as E11.5 with its expression steadily increasing until E16.5 (Gan et al. 1996; Xiang 1998). In 30% of RGCs, Brn-3a and Brn-3b are co-expressed although Brn-3a expression starts two days later, at E13.5 (Budhram-Mahadeo et al. 1995; Quina et al. 2005).

1.4.6 Role of the Brn-3b transcription factor in retinal ganglion cells

Brn-3b was initially identified following a specific search of POU domain transcription factors in mouse retina which led to the identification of a highly expressed

POUIV protein in the E16 embryonic retina (He et al. 1989; Xiang et al. 1993). Further studies revealed that this protein, Brn-3b or POU4F2, is expressed in retinal ganglion cells (RGCs) (Gan et al. 1996; Xiang 1998) and shares a high homology with the Brn-3a transcription factor (Ring et al. 1993). As shown in the figure below (figure 1.11), RGCs are born in mouse at embryonic stage E12.5 and continue to be produced until P0 when natural neuronal selection eliminates neurons which did not establish any synaptic connection with their targets (Erkman et al. 2000).

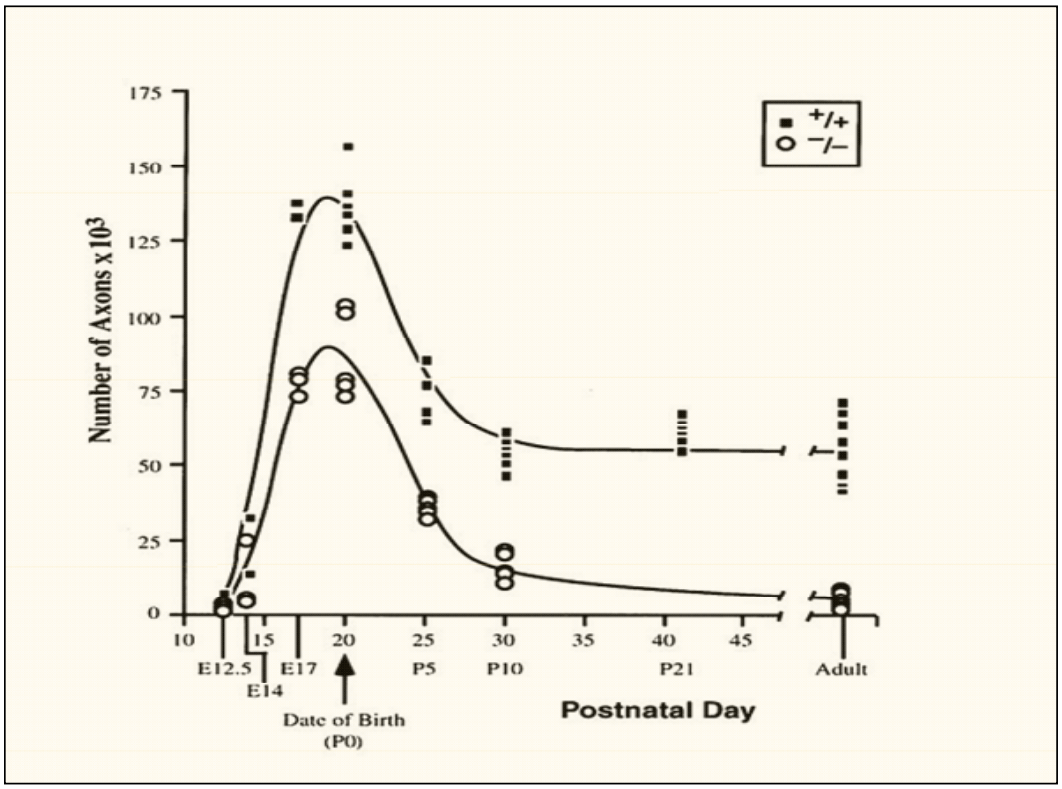

Fig. 1.10: Role of Brn-3b on the development of RGCs. Wild type or Brn-3b knock-out mice have been analysed for the number of axons produced by their respective retinal ganglion cells during their development. (Erkman et al. 2000)

As indirectly measured by the number of remaining axons, very few RGC survive in Brn-3b knock out mice (figure 1.10). Experimental evidence has shown that up to 70% of RGC are lost in Brn-3b knockout mice (Erkman et al. 2000). Since Brn-3b is expressed in circa 70% of RGCs (Erkman et al. 2000), this suggests that this transcription factor plays an essential role in all RGCs in which it is expressed. While the vast majority of RGC co-express Brn-3a, Brn-3b and Brn-3c (Pan et al. 2005; Badea et al. 2009), the lack of Brn-3b causes *per se* the death of RGCs (Erkman et al. 2000; Qiu et al. 2008). The remaining 30% of RGCs express Brn-3a and/or Brn-3c which partially compensate for the lack of Brn-3b (Xiang 1998). The experimental replacement of Brn-3b with exogenously transfected Brn-3a copies is associated with reduced RGC death levels (Pan et al. 2005). Once more this evidence suggests that Brn-3a is able to transactivate Brn-3b target genes which are involved in RGC survival (Pan et al. 2005). Furthermore, although with a much more severe effect, conditional Brn-3a knock-out mice show an impairment in dendritic stratification in the retina (Badea et al. 2009) suggesting that all Brn-3 transcription factors can have a functional role in RGC survival (Badea et al. 2009). RGC death in Brn-3a expressing cells, though, might be due to a temporal expression difference, whereas Brn-3a is expressed two days later and this might not be sufficient to rescue Brn-3b deficient cells from their death. (Qiu et al. 2008).

Brn-3b is also expressed in the superior colliculus (tectum in avian brain), the periaqueductal gray matter and in a small region of the brain stem (Xiang et al. 1993). The co-expression of the three Brn-3 transcription factors in these regions could also explain why trigeminal and dorsal root ganglia, where Brn-3b is also expressed, don't show any sign of neuronal loss in Brn-3b knockout mice (Ninkina et al. 1993). Although

the cause of RGC loss in mice lacking Brn-3b is not fully understood (Gan et al. 1996; Wang et al. 2000), anatomical evidence shows that these mice have a thinner retina and a reduction of the optic nerve diameter due to a dramatic loss of RGC axonal projection (Gan et al. 1996; Wang et al. 2000). Some of the remaining axons do not cross the optic chiasm and remain ipsilateral (Erkman et al. 2000). Mice lacking Brn-3b show a total abolishment of vertically moving stimuli, a reduction of the horizontally moving stimuli and a defect in pupil constriction in response to light stimuli (Badea et al. 2009). The death of Brn-3b knock-out RGCs is likely to be due to several causes including improper cell specification, differentiation and defects in RGC axonal pathfinding which results in their failure to enter the optic nerve (Erkman et al. 2000; Qiu et al. 2008). Furthermore, the forced over-expression of Brn-3b in non RGC-cells of the retina has the effect of repressing their growth (Qiu et al. 2008).

Several studies have partially unfolded the molecular events involved in RGC death (Wang et al. 2000; Wang et al. 2001; Weishaupt et al. 2005; Qiu et al. 2008). It has been established, for instance, that Brn-3b regulates the expression of other transcription factors such as Olf1, Brn-3a and Irx2 which are involved in neuronal differentiation (Mu et al. 2004). Other factors regulated by Brn-3b include proteins related to neuronal integrity such as gamma-synuclein, neurofilament light and middle chain (Mu et al. 2004). Overall though, the exact mechanisms leading to RGC death in Brn-3b knock-out mice are still being investigated.

1.5 Aim of the thesis

Given the importance of the Brn-3b transcription factor in the development of the retina (Turner et al. 1994; Xiang 1998; Gan et al. 1999; Erkman et al. 2000; Wang et al. 2000; DeCarvalho et al. 2004; Badea et al. 2009) and the potentially important role played by post-transcriptional gene regulation in neurons (Kim et al. 2004; Nelson et al. 2004; Rogelj et al. 2004; Sempere et al. 2004; Lai et al. 2005; Rogaev 2005; Smirnova et al. 2005), I wanted to analyse whether Brn-3b mRNA is subjected to post-transcriptional regulation in neuronal cells. As reported above, the 3'UTR of mRNAs plays a significant part in the regulation of mRNA stability, translation rate and turnover (Canete-Soler et al. 1998; Kenealy et al. 2000; Thames et al. 2000; Giles et al. 2003; Pontrelli et al. 2004; Andreassi et al. 2009; Ji et al. 2009). Intriguingly, not only the coding sequence, but also the 3'UTR of Brn-3b is highly evolutionary conserved (data presented in paragraph 3.0 of this thesis). This region exhibits between 99% and 97% nucleotide identity between human and mouse and mouse and rhesus monkey respectively (paragraph 3.0, figure 3.0), suggesting a possible important role due to its conservation. The same region of Brn-3a in contrast shows a 40% homology and the lack of any evident conserved regions (data not shown).

The first chapter of the results (chapter 3) will report the data obtained whilst investigating whether the 3'UTR of Brn-3b is subjected to post-transcriptional gene regulation in neuronal cells. For this purpose the 3'UTR of Brn-3b was sub-cloned in a reporter construct and the levels of the reporter mRNA and protein measured in ND7 cells subjected to differentiation. This has led to the identification of specific regions contained in its 3'UTR which are essential for its post-transcriptional regulation and

found that they are targeted by specific microRNAs, mir-23 and mir-214. The mutagenesis of their binding sites on the 3'UTR of Brn-3b has shown that these microRNAs play a direct role in the regulation of Brn-3b in neuronal cells. Furthermore these microRNAs and Dicer are up-regulated in ND7 cells induced to differentiate.

In the second results chapter of this thesis (chapter 4) it will be demonstrated that Brn-3b is differentially regulated by mir-23 and mir-214 in an immortalized retinal ganglion cell line (RGC-5). Contrary to what occurs in ND7 cells, Brn-3b levels are not affected by treatments aimed at inducing the differentiation of RGC-5 cells and one of the microRNAs involved in the regulation of Brn-3b (mir-214), is not expressed in the RGC-5 cell line. Intriguingly, mir-214 is not expressed in primary retinal ganglion cells. The co-transfection of mir-23 and mir-214 in RGC-5 cells is able to induce the reduction in the levels of both an exogenous construct containing the 3'UTR of Brn-3b and of the endogenous Brn-3b. The data presented in this chapter suggest that a pro-survival factor such as Brn-3b might be protected from microRNA-mediated regulation in RGC-5 in which it plays an essential role. This hypothesis is also supported by data in which the experimental knock-down of Brn-3b leads to increased RGC-5 cell death, thus paralleling a similar phenotype in primary RGCs.

In parallel to the analysis of the regulation of Brn-3b in immortalized neuronal cell lines, the role of Dicer in the differentiation of primary RGCs will be investigated.

CHAPTER 2

MATERIAL AND METHODS

2.1 Cell lines and transfection

The neuroblastoma ND7, the breast cancer MCF-7 and the RGC-5 retinal ganglion cell lines were routinely cultured in DMEM supplemented with 10% Fetal Calf Serum (FCS) at 37°C, 95% humidity and 5% CO_2. SH-SY5Y cells were cultured in 50% MEM, 50% Hams F12 supplemented with 15% FCS. All cell lines, apart from RGC-5 which was a kind gift of Professor Neeraj Agarwal (UNT Health Science Center, Fort Worth, TX, USA), are part of the laboratory stock. ND7 cells were produced by fusing primary rat dorsal root ganglion neurons with mouse C1300 neuroblastoma cells (Wood et al. 1990). All cells were grown to ~80% confluency prior to trypsinization which was performed by removing the old medium and gently washing the adherent cells with phosphate buffered saline (PBS) (Invitrogen) before adding enough trypsin (Invitrogen) to cover the cell monolayer. The flask was incubated at room temperature for 20-30 seconds, trypsin decanted and cells incubated for a further 40 seconds before being detached from the surface of the flask by pipetting with 5 ml of serum-containing medium (10% FCS) added to stop digestion by trypsin. Normally $1/10^{th}$ of the original culture was passed into a new flask.

Apart from RGC-5 cells, all cell lines were transfected with GeneJuice (Novagen) according to the manufacturer's instruction. RGC-5 cells were transfected with Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. For all the serum starvation experiments on the ND7 cell line, cells were transfected with the indicated constructs and the following day (day 0) incubated in full medium with or without FCS in order to induce differentiation for the indicated days. Staurosporine (Sigma) was diluted in dymethyl-sulfoxide (DMSO) and used at a final concentration of

20 μM. Actinomycin D (Sigma), which inhibits transcription by binding to the transcription initiation complex, was used at a final concentration of 7.5 μg/ml for 0, 4 and 8 hours (Giles et al. 2003). KCl was used at a final concentration of 50mM.

2.2 Cell storage and recovery

Cells were frozen by the following method. After trypsinization, cells were detached by repeatedly pipetting with Hanks Balanced Salt Solution (HBSS) and transferred to a 50 ml tube. Cells were then spun at 1000 RPM for 5 minutes, the supernatant decanted and the cell pellet was resuspended in 90% FCS plus 10% v/v dimethyl sulfoxide (DMSO, Sigma). Aliquots containing $1x10^6$ cells per ml were transferred to cryovials (NUNC, Roskilde, Denmark), placed into a freezing pot at -70^oC for 24 hours and then transferred to a permanent storage in liquid nitrogen. When needed cells were rapidly thawed at 37°C in a waterbath and transferred drop-wise to an appropriate flask with the appropriate pre-warmed growth medium. The following day the medium was replaced to eliminate any DMSO residue.

2.3 *E.coli* strains and transformation.

In this thesis the DH5α (F-, endA,1 glnV4,4 thi-1, relA1, gyrA96, deoR, nupG, lacZdeltaM15, hsdR17) and HM101 (F-, mcrB, mrr, hsdS20(rB-mB-), recA13, leuB6, ara-14, proA2, lacY1, galK2, xyl-5, mtl-1, rpsL20, glnV44) *E.coli* strains were used

(Stratagene). Cells were transformed according to the manufacturer's instructions. Briefly, 50µl of cells were transferred to a pre-chilled tube, mixed with the DNA to be transformed and incubated on ice for 30 minutes. Cells were then heat-shocked at 42°C for 42 seconds and quickly transferred back on ice for few minutes after which 750µl of LB medium (10g Tryptone, 5g yeast extract, 10g NaCl in a liter of dH_2O) was added and cells transferred into an orbital incubator for an hour at 37°C. After incubation, 250µl of cells were plated onto LB-agar plates containing the appropriate antibiotic, spread with a glass rod and incubated overnight at 37°C. Colonies were then individually picked with the end of a yellow tip, transferred into a 15ml tube containing LB supplemented with the appropriate antibiotic and incubated overnight at 37°C. The following day an aliquot of cells was subjected to a small scale extraction of DNA with the aid of a kit (Qiagen). In this protocol, an aliquot of the overnight bacterial growth is transferred to a 1.5ml tube and bacteria spun at 10.000rpm for 40 seconds. The supernatant is discarded and bacteria are resuspended in 100µl of solution 1 (50 mM glucose, 25 mM Tris-Cl, pH 8.0, 10 mM EDTA, pH 8.0) then incubated for few minutes in 100µl of solution 2 (0.2 N NaOH, 1% SDS) and then in 250µl of solution 3 (3 M potassium acetate, 11.5% acetic acid). Following a spin at 13.000rpm for 10 minutes, the supernatant containing the DNA is bound to a silica-based column, washed with 750µl of solution QF and eluted with 50µl of ddH_2O. DNA is then quantified via the Nanodrop spectrophotometer (Thermo Scientific). A larger scale of the above mentioned protocol (maxiprep) was routinely used when larger amount of plasmid DNA were needed.

2.4 Site-directed mutagensis

Mutation of the microRNA target sequence on the 3'UTR of Brn-3b was achieved via the QuickChange site directed mutagenesis (Stratagene) following the manufacturer's instructions with the following primers:

Mir-23 upstream	Fw-5′ AACAATTCCGGTAAACGGGCACCCAGACCAAGCCAG 3' Rw 5′ CTGGCTTGGTCTGGGTGCCCGTTTACCGGAATTGTT 3'
Mir 23 downstream	FW-5′ GAGTTGATGCTTAACGGGCTATGATAGAGACATCTC 3′ RW-5′ GAGATGTCTCTATCATAGCCCGTTAAGCATCAACTC 3'
Mir 214	Fw 5′ GGAAGTTGCGACGGTTGCGTTCACTG 3' Rw 5′CAGTGAACGCAACCGTCGCAACTTCC 3'
Mir128	Fw 5′ CCGTGTAGACCAGATGCGCTGGCGGAAGTC 3' Rw 5′ CAAGACTTCCGCCAGCGCATCTG GTCTACA 3'
Mir-323	Fw 5′ AGAGTTGATGCTTCAGGTGCTATGATAGAG 3' Rw 5′ GTCTCTATCATAGCACCTGAAGCATCAACT 3'

Table 2.0: Primers used for mutagenesis. Primers used for the sited directed mutagenesis of the reported microRNA binding sites on the 3'UTR of Brn-3b.

2.5 Deletion of the GFP::3b-3'UTR with *Bal-31*

The sequential deletion of Brn-3b was performed with the *Bal-31* nuclease (New England Biolabs). Firstly 10µg of GFP::3b-3'UTR construct were linearized by digesting with the *PsiI* restriction enzyme (Promega). The linearized DNA was then added to a master mix containing the *Bal-31* buffer and *Bal-31* enzyme. Aliquots were then transferred to eight 1.5ml tubes kept on ice. At time zero the tubes were transferred to a 30°C water bath and the reaction stopped at different time points by adding EDTA to a 0.05M final concentration and by heating at 75°C.

2.6 Coverslip preparation

Glass coverslips (13mm, VWR International) were etched for 30 minutes in nitric acid in a 50ml tube at room temperature, rinsed several times with ddH_2O and incubated in absolute methanol for 15'. Coverslips were then spread on a piece of 3MM paper and allowed to dry in an oven (or room temperature) after which they can be used immediately or stored at room temperature indefinitely. On the day of use, coverslips were spread on a square piece of 3MM paper and 100µl of Poly L-Lysine (PLL) (200ng/µl) was added on one side and incubated for 30 minutes after which PLL was eliminated and coverslips briefly rinsed in ddH_2O. 100µl of Laminin (50 ng/µl) were subsequently added to the PLL-treated side, incubated for 30 minutes, rinsed with ddH_2O and allowed to dry at room temperature.

2.7 Chick retina isolation and culture:

Fertilized Bovans Goldline eggs were obtained from Henry Stewart & Co.Ltd and incubated at 37.5°C in 90% humidity. Embryos were staged according to Hamburger and Hamilton (Hamburger 1951; Hamburger et al. 1992). At the desired developmental stage (E6, E7 and E8) eggs were removed from incubator, a circular aperture opened on top of the shell with scissors and the chick embryo isolated and sacrificed. The head, separated from the body, was transferred into a 10 ml petri dish containing HBSS or PBS. Under a microscope (STEMI SV11+Zeiss Schott light, KL1500 electronics), the eyes were carefully isolated from the rest of the tissue. The connective tissue and then the pigmented epithelium were carefully removed paying attention to avoid the piercing of the retina, and then the iris and the corpus vitreum were removed. The isolated retina of

circa 0.5 cm^2 was then flat-mounted, retinal ganglion cell side-up, on a piece of nitrocellulose filter (Sartorius AG) pre-wet in HBSS or PBS as shown in figure 2.0.

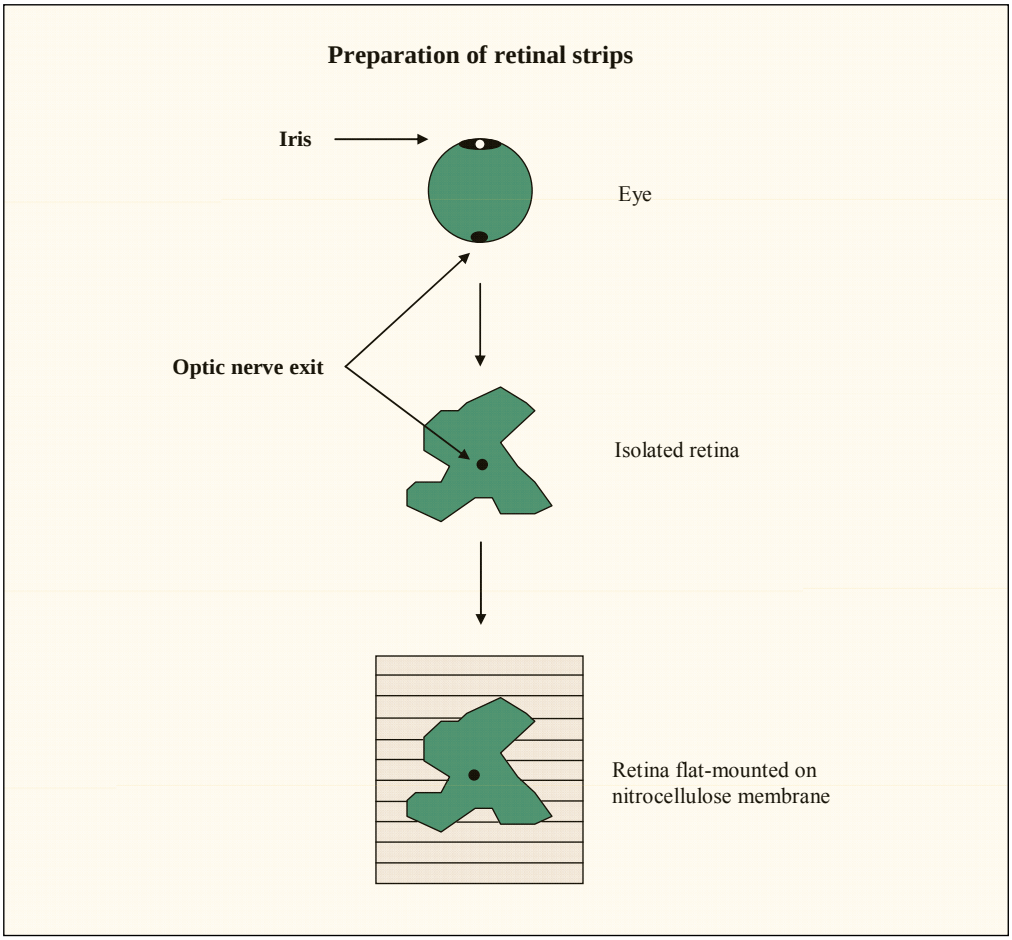

Fig. 2.0: Preparation of retinal strip. See section 2.7 for explanation**.**

The filter with the attached retina was then transferred to the "chopping" board of a tissue chopper (Mc Ilwain) and cut in strips of the desired width (normally between 100 and 200 μm) while keeping the tissue moist with full medium (DMEM, 10% FCS, 2% Chicken serum, 100U/ml Penicillin/100μg/ml Streptomycin (Invitrogen). Following the cut, strips were individually picked with the aid of tweezers and rapidly transferred,

(retina facing down) onto pre-wet PLL/Laminin coated coverslips, each set in the well of a six-well culture dish. Normally two strips were laid on coverslips in parallel 5-6 mm one from the other and a light stainless steel bar was set transversally on top of the strips to stop them from moving (figure 2.1). One or two drops of full medium were then added on top of the coverslips and then incubated for ten minutes at 37°C before 4ml of full medium were added and incubated at 37°C and 5% CO_2 in a humidified incubator (Jencons-PLS).

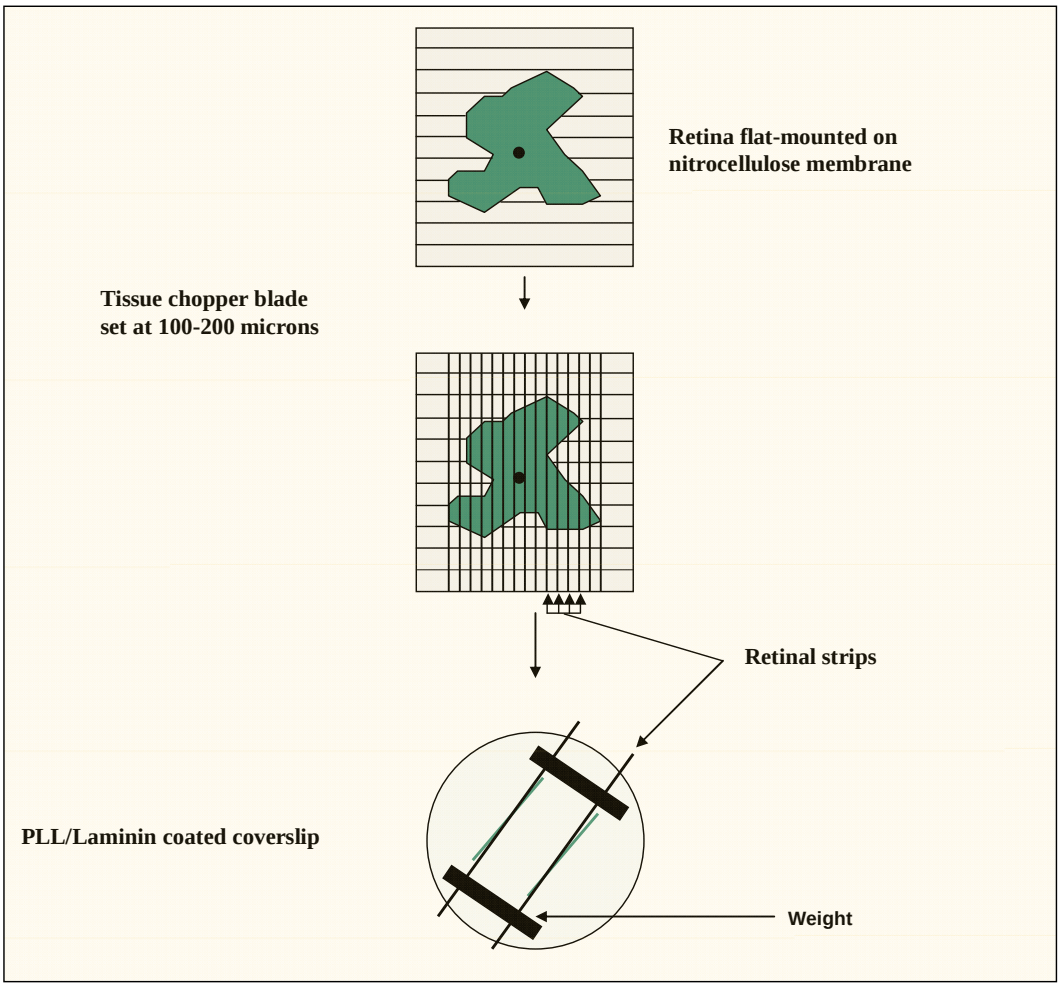

Fig. 2.0: Preparation of retinal strips and assembly on PLL/Laminin coated coverslips.

2.8 Immunofluorescence of primary RGCs

For the analysis of the expression of Dicer in primary chick retinal ganglion cell neurites, retinal strips were grown on coverslips as described above and after 4 days processed for immunofluorescence analysis. Paraformaldheyde (PFA) at 4% in PBS (w/v) or -20^{o} C cold methanol, were used to fix/permeabilize RGCs the detection of actin (PFA) and Dicer (methanol). PFA was dissolved in 60-70°C hot water in the presence of alkali. The solution was then filtered and either used immediately or aliquoted and stored at

-20°C. Methanol was stored at -20°C for at least 2 hours before use. Coverslips were transferred to individual wells of a 24-well plate with the retinal strips facing up and immediately incubated with 500µl of the fixative of choice for five minutes. After methanol or PFA were removed, coverslips were submerged in 1X PBS until further use. Coverslips fixed with PFA were subsequently permeabilized with 0.1% Triton-X100/PBS for 5 minutes at room temperature. Methanol has both fixative and permeabilization properties and thus the treatment with Triton-X100 is not necessary.

Following the fixative-permeabilization step, anti-Dicer (Santa Cruz, SC30226) or anti-Actin (Abcam Ab40864) antibodies were diluted 1:25 or 1:200 respectively in 1%BSA in PBS and 50µl were spotted in the well of a six-well plate. Coverslips were then lowered on the drop containing the antibody dilution, retina facing down, the plate sealed and incubated overnight at 4°C in a humidified chamber to avoid evaporation. The following day coverslips were rinsed three times with PBS and then incubated with a 1:500 dilution of anti-Rabbit-FITC conjugated (DAKO) in 1%BSA/PBS for 1 hour at room temperature followed by two brief washes with PBS and briefly rinsed with ddH2O

to remove salts. The coverslips were then lowered, retina-side facing down, on a drop of mounting medium containing DAPI (DAKO) on the surface of a microscope slide. When DAPI is bound to DNA and is excited with ultra-violet light it emits a violet light which is used to localize cell nuclei. Coverslips were then sealed with nail-varnish which was allowed to dry in the dark for twenty minutes before analyzing them under fluorescent and/or light microscope.

2.9 RNA extraction

Growth medium was removed and cells were washed twice with PBS before between 500µl and 1000µl of Trizol (Invitrogen) solution was added to each individual well according to its size. Cells were incubated in this solution for 2-3 minutes at room temperature. A syringe plunger was used to scrape the resultant cell lysate from the plate surface and then pipetted into a 1.5ml tube and 200µl of chloroform added. The lysate was shaken vigorously for 30 seconds, incubated at room temperature for 5 minutes and spun at 12,000g in a refrigerated centrifuge at 4^{o}C for 15 minutes. The aqueous supernatant was transferred to a new 1.5ml tube and 500µl of isopropanol added. The lysate was then incubated for 10 minutes at room temperature before the mixture was spun at 12,000g in a refrigerated centrifuge at 4^{o}C for 15 minutes. All liquid was removed from the tube and the resultant RNA pellet washed once with 70% ethanol before being spun at 12,000g (at 4^{o}C) for 5 minutes. All liquid was again removed from the tube by pipetting and the RNA pellet dried at 60^{o}C for few minutes. The RNA was then resuspended in an appropriate volume of sterile distilled water and stored at -20^{o}C until use.

Following extraction, the quality of the total RNA was evaluated via spectrophotometric analysis. Good quality RNA has an A260/A280 ratio close to 2.0 (Sambrook *et al.,* 1989). RNA was also visually inspected by running an aliquot on a 1.0 % agarose gel in a Tris-Acetate-EDTA medium (40 mM Tris base, 40 mM Acetic acid, 1 mM EDTA) containing 0.8 μg/ml ethidium bromide. Intact RNA shows a 28S ribosomal RNA band of circa double the intensity of the 18S ribosomal RNA band when viewed under ultraviolet radiation.

2.10 Synthesis of single-stranded copy DNA (cDNA)

RT-PCR is performed on a DNA template, so extracted RNA is reverse-transcribed to complementary DNA, (cDNA) prior to PCR amplification. This was performed using a commercial reverse-transcriptase enzyme, Superscript II (Invitrogen). The reaction was primed random hexamers (GE Healthcare, UK). Reactions were performed according to the manufacturer's general instructions. Generally, 1μg of total RNA was incubated with 250ng of random hexamers, and the total volume made up to 11μl with sterile distilled water. This was then placed in a 70^{o}C water-bath for 10 minutes to denature any RNA secondary structure and subsequently transferred on ice. After few minutes, an 8 μl mix, containing 2μl 0.1M DTT (Invitrogen), 4μl 5x Superscript II First Strand Buffer (Invitrogen), 1μl 10mM dNTP mix (Promega), 1μl (24.5 units) RNaseOUT (an RNase inhibitor; Invitrogen) and 1μl (200 units) of Superscript II RT enzyme were added to the denatured RNA and incubated at 42^{o}C for 1 hour. Following the reaction,

80µl sterile distilled water were then added to bring the final volume to 100µl. cDNA samples were subsequently stored at -20°C until use.

2.11 Real time RT-PCR primer design

RT-PCR primers were designed according to available sequences. They were tested for an optimal annealing temperature of at least 60°C, the lack of any evident hairpin formation, self-annealing and formation of primer-dimers. A list of the primers used in this thesis is reported in the table below (table 2.1).

	Target	Primer names	Primer sequences 5'-3'	Annea-ling T (°C)	Amplicon size (nt)
	β–actin	Actin F Actin R	5'-AGA TGA CCC AGA TCA TGT TTG AG-3' 5'-AGG TCC AGA CGC AGG ATG-3'	**60**	**187**
	3b-3'UTR	3b-Sac F 3b-Bam R	5'GAGCTCTAGGGACCCTTCTCCAGGGATGGCCC T-3' 5'GGAACCCGGGTGCCAGTAAAACATTAAAA TCATCTC3'	**60**	**1750**
	MAP2C	MAP2C F MAP2C R	5'-CACAAGGATCAGCCTGCAGCTCG 3' 5'-TTC TGG CTC CTC CTC AGTCA 3'	**60**	**353**
	Dicer	Dcr F Dcr R	5'- GGGGCGCATAGTAGGTTCTGCTCACGCC 3' 5' CCTTCGAGAACCATCGGACCCCGCTCCG -3'	**60**	**492**
	GAPDH	GAPDH F GAPDH R	5' GTGCGAACGGATTTGGACG 3' 5' CCAGTAGACTCCACGACACA 3'	**60**	**472**
	Brn-3b	Brn-3b F Brn-3b R	5'-AGCCGGCTCCCCCTTTCA-3 5'-GACGGCCCATATCTTCTTC-3	**59**	**455**
	Brn-3a	Brn-3a F Brn-3b R	5'-TGGCGTCCATCTGCGACTC- 3' 5'- CTCAGGCTTGTTCATTTTCTC- 3'	**60**	**280**
	MAP2C (Chick)	MAP2C F MAP2C R	5' AGAGGGCGCCTTGCTTCCCC 3' 5' TGCCAGGGCTGTGCTGCTGT 3'	**60**	270

Table 2.0: List of primers used in this thesis

2.12 Real time RT-PCR

In this thesis, changes in gene expression on target cells were measured by quantitative real-time reverse-transcription polymerase chain reaction (qRT-PCR). Real-time qRT-PCR is a technique for quantifying the amount of mRNA expression of a particular gene at a particular time or in a particular cell or tissue type. It relies on the fact that PCR amplifies a target in an almost exponential manner until the reaction reaches a plateau phase due to limiting conditions, such as primer availability. By monitoring the accumulation of product in real-time after each amplification cycle (using fluorescent dyes), the exponential/non-exponential status of the reaction can be determined and the amount of starting template in two samples relative to each other can be calculated by a variety of analyses (in this thesis, the $2^{-\Delta\Delta Ct}$ method was used). Subsequently, some of the mRNA expression differences observed were also investigated at the level of protein expression.

Quantitative RT-PCR (qRT-PCR) was performed using the DNA Engine Opticon system (MJ Research). Briefly, PCR reactions were performed in duplicate in white-coloured plastic 8-well strips or in white-coloured 96-well plates (both from BioRad) in a final volume of 20µl, containing 5µl of cDNA, 500 nM (0.5µM) of each specific primer and 1x Platinum SYBR Green qPCR mix (Invitrogen), according to the manufacturer's instructions. Amplification was via an initial denaturation step at 95^{o}C for 3 minutes to activate the Platinum hot-start Taq, followed by cycling of 95^{o}C for 30 seconds, 57-62^{o}C (depending on the primer pair) for 30 seconds and 72^{o}C for 30 seconds. A final melt curve analysis was carried out routinely from 65^{o}C to 95^{o}C with 0.3^{o}C steps. When the melting temperature of a specific amplicon is reached the two strands separate, the SYBR I Green

dye is released and therefore no longer fluoresces. Thus melt curves are used to verify product composition. In addition, an aliquot of the reaction was run on agarose gel containing ethidium bromide in order to visualise the products and verify their size. As a negative control, a minus reverse-transcriptase and a minus cDNA samples were added for every PCR reaction. The first negative control monitors the exclusion of any DNA carry-over during the extraction of RNA. The second controls for the exclusion of any DNA contamination from pipettes, water, buffers etc.

In order to evaluate the amount of a specific amplicon, the $2^{-\Delta\Delta Ct}$ method (Livak & Schmittgen, 2001) was used. This relies on the "normalization" of the amplified product versus a control gene whose expression does not vary in those specific experimental conditions. This allows for the exclusion of variables including sample-to-sample differences in RNA input, RNA quality and reverse-transcription efficiency that might contribute to apparent inter-sample target gene expression differences. Expression of these normalizing genes could then be used in analyses to 'equilibrate' differences due to non-biological variables. After some tests, the β–actin, and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) normalizing genes were chosen. Following the RT-PCR with the Opticon system, raw data were processed via the $2^{-\Delta\Delta Ct}$ to evaluate the expression levels of the mRNAs.

2.13 Polymerase Chain Reaction

Polymerase chain reaction (PCR) is a technique which allows the exponential amplification of a specific stretch of DNA by using a thermostable DNA polymerase and

DNA oligonucleotides as primers. A non-quantitative PCR was used to amplify the 3'UTR of Brn-3b (paragraph 3.1 of this thesis) by using 50 ng of mouse chromosomal DNA, 50 μM dNTPS, 3 mM $MgCl_2$, and 20 pmoles of each oligos. A denaturation step at 94°C for 30" was followed by the annealing step at 55°C for 30", followed by an extension step at 72°C 1'30". The cycles were repeated 30 times and an aliquot of the PCR run on an agarose gel to be analyzed before being processed for its subclonin as described in paragraph 2.17.

2.14 Precautions in manipulating nucleic acids

RT-PCR is a powerful technique, capable of amplifying millions of amplicons from just a few template molecules. Consequently, small levels of DNA contamination (for example, from previous PCR amplifications and samples with high DNA levels) can easily result in product formation even in the absence of added cDNA template. All RT-PCRs in this project were performed simultaneously with "no template control" reactions (in which sterile distilled water rather than a cDNA template was added) in order to verify that positive results were not caused by contamination of the reaction by an external DNA source. However, in order to minimise the possibility of contamination, a number of precautions were taken at all steps prior to (RNA extraction and cDNA synthesis), and including, the PCR:

1. Use of dedicated equipment and consumables for RNA extractions, cDNA synthesis and RT-PCRs only (pipettes, tubes, tube racks, solutions).

2. Use of sterile filtered tips to prevent contamination of dispensing pipettes by aerosols.
3. Use of gloves and frequent glove changes.
4. Aliquoting of solutions (dNTPs, primers, Taq DNA polymerase buffer) using dedicated pipettes to prevent contamination of original stocks.
5. Use of ultraviolet radiation (UV)-treated sterile distilled water aliquots.
6. Physical separation of pre- and post-PCR manipulations.

2.15 Northern blot

For northern blot analysis of microRNAs, total RNA was extracted as described above and its concentration evaluated via Nanodrop (Thermo Scientific). Thirty micrograms of total RNA were resuspended in an equal amount of 2X RNA sample buffer (720µl of 37% formaldehyde (12.3M), 2ml of 100% glycerol, 3084µl of formamide), heated at 70°C for 10 minutes, cooled on ice and then loaded onto a 15% polyacrylamide, 7M Urea, 1X TBE gel which had been pre-run for 10 minutes at 200volts. Samples were then run at 200volts in 1X TBE until the bromophenol blue dye had reached the bottom of the gel. The gel apparatus was then dismantled and the nucleic acids transferred to a Hybond N+ (Hybond) membrane via a semi-dry apparatus at 10volts for 20 minutes in 0.5X TBE buffer. The nucleic acids were subsequently immobilized onto the membrane in a UV-crosslinker set at 1.200 joules (BDH). The membrane was then pre-incubated in a 50ml falcon tube with 5ml of Ultra-Hyb oligo hybridization buffer (Ambion) at 42°C for two hours. The pre-hybridized membrane was

then incubated overnight in 5ml of pre-heated Ultra-Hyb oligo hybridization buffer containing the appropriate probes at 42°C.

Probes:

U6: gcaggggccatgctaatcttctctgtatcg

mir-214: ctgcctgtctgtgcctgctgt

mir-128: aaaagagaccggttcactgtga

mir-23: ggaaatccctggcaatgtgat,

were end labeled for an hour at 37°C with T4 polynucleotide Kinase (Promega) in the presence of 5µl of γ^{32}P-ATP (50uCi or 1.85MBq). The following day the membrane was washed three times for 1 minute each at room temperature in 2×SSC, 0.1%SDS. Membranes were sealed in a plastic bag and then exposed to a phosphor-imager screen (Biorad). Following exposure for the desired amount of time the screens were scanned via a phosphor-imager machine (Biorad) and bands quantified with the QuantityOne software (Biorad).

2.16 Western blot

For western blot analysis, cells were normally grown in a six-well plate. On the day of the experiment, cells were washed twice with PBS and lysed with 100µl of RIPA buffer (10mM TRIS pH 7.5, 150mM NaCl, 0.2% NP-40) supplemented with protease inhibitors (mini-cocktail, Roche). The cell lysate was collected, transferred to a 1.5ml tube and spun at 13.000 rpm for 5 minutes to remove cell membranes. An aliquot of the cell lysate was transferred into a new 1.5 ml tube and mixed with the appropriate amount

of 4X Laemmli sample buffer (0.25M Tris, pH 6.8, 6 % SDS, 40 % glycerol, 0.04 % Bromophenol Blue, 0.04% xylene cyanol, 20%β -mercaptoethanol (β–ME).

Samples were then incubated at 95°C for 3-5 minutes to denature proteins and subsequently loaded on polyacrylamide gel for proteins. This gel is composed of two parts which are polymerized sequentially one on top of the other. The lower gel or resolving gel is composed in accordance to the desired acrylamide concentration as exemplified in the table below (table 2.2):

% Total Acrylamide:		**8 %**	**10 %**	**12 %**	**15 %**
H_2O	(ml)	12.1	10.5	8.75	6.25
4X Tris Buffer Ph 8.8*	(ml)	6.25	6.25	6.25	6.25
29:1 Acrylamide:Bis	(ml)	6.67	8.35	10	12.5
10 % APS	(λ)	130	130	130	130
TEMED	(λ)	14	14	14	14
Total Vol:		25 ml	25 ml	25 ml	25 ml

Table 2.2: Lower (resolving) gel composition *4X Tris Lower Buffer: Tris-base 1.5M, Sodium Dodecyl Sulfate (SDS) 0.4%, pH to 8.8 with HCl

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An increase in acrylamide concentration allows a better separation of low molecular weight protein and vice versa. After pouring the gel, a layer of butanol or isopropanol was slowly layered on top to reduce the contact with air (which reduces the polymerization rate) and to level the surface. When the gel was polymerized, butanol was removed, the surface gently washed with ddH2O, air-dried and the upper gel (or stacking gel) added on top with a comb to create the wells (table 2.3).

% Total Acrylamide:		5 %
H_2O	(ml)	5.8
4X Upper Tris Buffer*	(ml)	2.5
29:1 Acrylamide:Bis	(ml)	1.7
10 % APS	(λ)	40
TEMED	(λ)	20
Total Vol:		10 ml

Table 2.3: Upper (stacking) gel composition.*4X Tris Upper Buffer: Tris base 0.5M, SDS 0.4%, pH to 6.8 with HCl

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Following polymerization the gel was submerged in running buffer (25mM Tris-Cl, 190mM Glycine, 0.1%SDS), the comb carefully removed, the wells flushed once or twice with running buffer to eliminate any non polymerized acrylamide and samples loaded in the wells. A current of 25mA was applied and samples run until the bromophenol blue reached the lower end of the gel. Proteins were then transferred onto Hybond-C nitrocellulose membrane via a semidry apparatus (Biorad) at 10V for 30 minutes. The transfer occurred in the presence of 1X transfer buffer (3.03gr Tris, 14.4gr Glycine, 20ml Methanol brought to a liter with ddH_2O). After the transfer, the membrane was visually inspected to assess the transfer of the protein marker and then incubated in 5% non-fat milk, 0.1% Tween-20 in PBS for an hour or more. This step blocks any non specific sites on the membrane. Subsequently the membrane was incubated for an hour with the appropriate primary antibody, washed twice with milk-tween20, incubated with the secondary antibody conjugated to horseradish peroxidase (HRP), washed twice with

PBS-tween-20 and incubated for 1 minute with a 50-50 mix of chemiluminescence solution A and B (Pierce). The solution was then eliminated and the membrane exposed to autoradiography films (Kodak) for the necessary time. Films were then developed using an automated developer (Xograph Compact X4). Antibodies used: anti-GFP 1:5000 (Sigma G-1544), anti-Neomycin 1:1000 (Upstate), anti-P85 1:1000 (Upstate), anti-Dicer 1:250 (Santa Cruz, SC30226).

2.17 Constructs and vectors

The 3′UTR of Brn-3b (NM_138944) was amplified via polymerase chain reaction (PCR) from 500ng of mouse genomic DNA with the following primers:

3bUTR-SacI: 5′gagctctagggacccttctccagggatggccc3′ and

3bUTR-BamHI: 5′ggaacccgggtgccagtaaaacattaaaatcatctc3′.

The resulting product of circa 1.7Kb was sub-cloned in the *SacI* and *BamHI* multiple cloning site of the pEGFP-C1 vector, downstream of GFP. Following the ligation, the product was transformed in DH5α competent cells as described in section 2.3, page 64 of this thesis. The mCherry vector (Clontech) encoding for red fluorescent protein was part of the lab stock

The Brn-3b shRNAmir-pGIPZ clones (V2LHS_47689, V2LHS_47684, V2LHS_47687) were purchased from Open Biosystems. The vector used for the over-expression of Dicer in primary RGC (pUHD10-3) is a kind gift of Professor Tatsuo Fukagawa Department of Molecular Genetics, National Institute of Genetics, Japan.

2.18 Measurement of cell death via flow cytometry analysis

RGC-5 and ND7 cells were transfected with shRNAmir as described in section 2.1, their medium changed after four hours and then incubated in full medium for two days. The supernatant and trypsinized cells of each sample were incubated with propidium iodide and run according to standard operating procedures on a LSRII analyser (BD bioscience) using FACSdiva® software. Cells were gated according to their light scattering properties. For analysis of GFP-labelled cells, live cells were plotted showing staining on GFP-A channel and PI-A (propidium Iodide). Typically 20,000 events were collected in each FACS experiment which was repeated at least four times. Error bars indicate standard deviation.

2.19 MicroRNAs and siRNA

MicroRNAs (mir-23 and mir-214) were ordered from Applied biosystem and had the following sequence:

Mir-23: AUCACAUUGCCAGGGAUUUCC

Mir-214: ACAGCAGGCACAGACAGGCAGU

These were transfected in RGC-5 cells, ND7 cells and in primary retinal cells with Lipofectamine 2000 (RGC-5 and primary RGCs) and Genejuice (ND7) at a 50μM final concentration as described previously. The siRNA specific for Dicer was ordered from Applied biosystems (s23755) and transfected with Lipofectamine 2000.

2.20 Measurement of protein concentration

The BCA assay (Smith *et al.*, 1985) measures the formation of Cu^{+} from Cu^{2+} by the biuret complex in alkaline solutions of protein using bicinchoninic acid. The BCA reagent forms a complex with Cu^{+}, which has a strong absorbance at 562 nm. Protein for the standard curve used was BSA (Sigma) 1mg/ml, concentrations for the standard curve range from 0 (blank) to 50µg. 5µl of buffer (RPMI) was added to the standard curve samples to account for any changes in absorbance caused by RPMI. 5µl of samples were added to 45µl of water. 1ml of BCA (sigma) was added to each sample (including standard curve samples) and mixed. The mixture was incubated in water bath at 37^{o}C for 10 minutes following which 20µl of copper sulphate solution were added to each sample. All samples were incubated at 37^{o}C for at least 20 minutes. Following incubation, samples were cooled at room temperature and their absorbances measured at 562 nm versus a blank. The concentration of unknowns were then determined from a plot of concentration vs. absorbances obtained for the standard protein solutions.

2.21 Statistical analysis

Paired T tests were used to analyse the data. Real time RT-PCR data were analysed using one-way Anova followed by Dunnett's multiple comparison test (if compared to control sample) or Bonferroni's *post hoc* test (if compared to one another). P values of < 0.05 were considered statistically significant.

Chapter 3

The expression of Brn-3b is regulated by specific microRNAs in neuronal cell lines

3.0 Evolutionary conservation of the 3'UTR of Brn-3b

Members of the POUIV family of transcription factors show some intriguing aspects at the level of their evolutionary conservation. As one would expect from members of the same class, the coding sequence of Brn-3a and Brn-3b is more than 95% homologous (data not shown).

Differences are found instead at the level of their 3' untranslated region (3'UTR) whereby this region between Brn-3a and Brn-3b is only 35% homologous (data not shown). Similarly the 3'UTR of Brn-3a, with a 35% homology, is poorly conserved amongst different species (data not shown) while, in contrast, the 3'UTR of Brn-3b in mouse, rat and human is more than 60% homologous (figure 3.0).

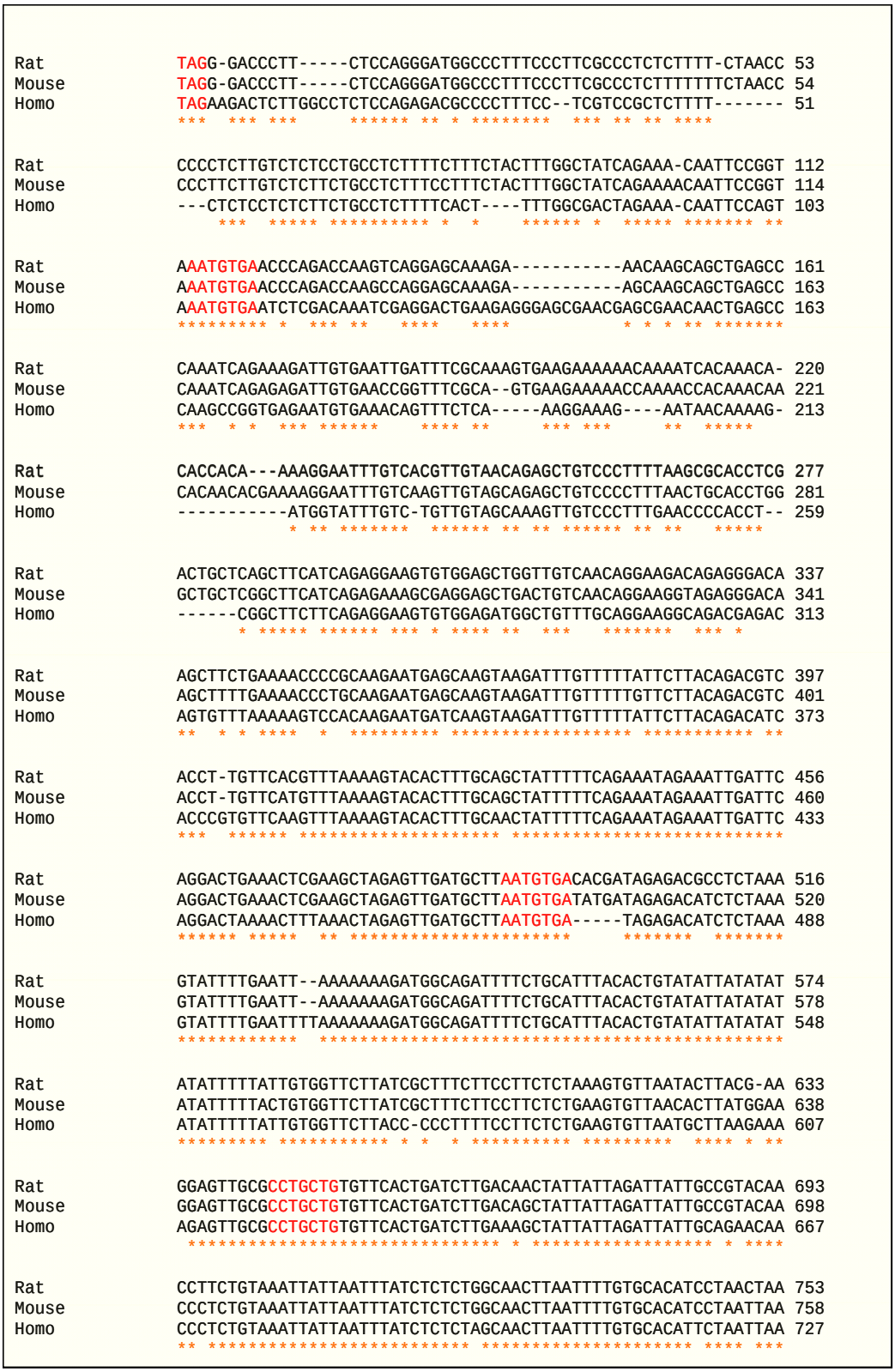

Fig. 3.0: Alignment of the 3'UTR of Rat, Mouse and Human Brn-3b. The 3'UTR of rat, mouse and human Brn-3b (XM_001074669 NM_138944, NM_004575.2 respectively) were aligned via bioinformatic tool. Red boxes indicate the binding sites of the following microRNAs, from top to bottom: mir-23 (upstream), mir-23 (downstream), mir-214, respectively. The TAG at position 1 indicates the stop codon.

```
Rat             CTAAACTTCTTTAGTCTGAAAAATAAAACCGGCGGATACATATAGGTAGTGATGCTCAAA 813
Mouse           TTAAACTTCTTTAGTCTGAAAAATAAAACCGGCGGATACGTATAGCTAGTGATGCTCAAA 818
Homo            TTAAACTTCTTCCGTCTAAAAAA----AGTGGGGGAAATGTATAGCTAGTAACGTTCAAA 783
                 **********  **** *****    *  ** *** *  ***** **** * * *****

Rat             TGTTCTTTTTTCTTTTTCCGTCTGATGAGTTCCCTGGAGTTTCACAGCTTTCCCGCTTAG 873
Mouse           -ACATTTTTCTTTTTTTCTTTTTGGT------CCTGGATTTTTACAGCTT-CCCACTTAG 870
Homo            ---AAATTTTGTTTGATGAGTTTACC---------GAATTTTTACAGCTT-TCCTCCTAT 830
                      ***   **  *   * *            * * *** *******  ** * **

Rat             ACTGTGTTCTCTTCGGCCTAGTTGTATATTCTCACTTTGAATGAAGATTGTTTCCTCCCT 933
Mouse           ACTGTGTTCGCTTTGGCCTAGTTGTATATTCTCACTTTGAATGAAGATTGTTTCTTCCCT 930
Homo            ACTGTGTTCCTTTTGACCCATTTGTATATTCTCACTT-GAATGAAGATTGTTT-TTTTCT 888
                *********  ** * ** * ***************** ***************  *  **

Rat             TTGTTTTTAACTGGTAGCGTTCTAATTTGTGAGCTGACACTTGATAATGGATGTCTTAAT 993
Mouse           TTGTTTTTGACTGGTAGTGATCTAATTTGTGAGCTGACACTTTATAATTGATGTCTTAAC 990
Homo            TTGTTTTT-ACTGGTAGTGTTCTGATTTGTGAGTCGACACTCAGTAATGGATGTCTTAAT 947
                ******** ******** * *** *********  ******   **** **********

Rat             CGTGTAGATCTGATTCACTGTCCGAAGTCTTGTTTACTTCCTTCCATATTTAATGGGGAT 1053
Mouse           CGTGTAGACCAGATTCACTGTCCGAAGTCTTGTTTACTTCCTTACATATTTAATGGGGAT 1050
Homo            CGTGTAGACCTGATTCACTGTCTGAAGTATTGTTTACTTCGTTACATATTTAATGGGGAT 1007
                ******** * ********** ***** ********** ** *****************

Rat             TCCCACATTGTCCCCCGCAGCACACGTGCTCTCTCACTCACTTTACAAGCCTACACACAG 1113
Mouse           TCCCATATTGTCCCC-GCGGCACACGTGCTCTCTCACTCACCTTACATGCCTACACACAA 1109
Homo            TCCCACATTGTCCCC-ATGACACATGAGCGCTCTCACTTACCCT---TACACACACACAC 1063
                ***** *********     **** * ** ******** **  *     *  *******

Rat             ACACACAGTACACGTCTCTAACAGAAGAGAAGGGGCAGTTGGAGGTACGACTGATAACCA 1173
Mouse           ACACACA-TACACATCTCTAACAGAAGAGAAGAAGCAGCTGGAGGTACCAACGATGAGCA 1168
Homo            ACACACA-CACACACCTCTAACAGAAGGGAAGAAGCAGTTGGAAGCATGACCGAT----- 1117
                *******  ****  ************ ****  **** **** * *  *  ***

Rat             GTGTATCCTTTTCCAGCCT-AGGTGCGTTCTCCACTTGGTGCTTACTCTGCTGGACTCAA 1232
Mouse           GTGTATACTTTCCCAGCCTTAGGTGCAGTCTCCACTTGGTGCTTACTCTGCTGGATTCTA 1228
Homo            --GCACCATTTTCTAGTTTTAGGTGCATTTGCCACTTGGTGTTTGCCCTTCAGATTTT-- 1173
                  * *   *** * **  * ******  *  ********** ** * ** * *   *

Rat             AGATTTCACCCAGGTATTTCAATCTCCCAGCTTTTAGTTGCTTTGT-GGCTACATTT-GA 1290
Mouse           AGATTTCACCAAGGTATTTCCATCTCCCAGTTTTCGATTGCTTTGT-TGCTACATTTTGA 1287
Homo            AGATTTCACCAAGGTATTTCAGTCTTCCAGTTTTCAATTGCTTTGTTGGCTACATGTTAA 1233
                ********** *********  *** **** ***   *********  ******* *  *

Rat             CCTTTATAGGA-TCCTTCAGTTTTTCCTTTTAGGATGTTTGTTTGTAGGGG-AAAATTTT 1348
Mouse           CCTTTATAGGGGTCCTTCATTTTTTCCTTTTAGGAGGTTTGTTTGTAGGGGGAAAAATTT 1347
Homo            TATTTATAGGAATACTTCAGTTTTTCCTTTT-GGAGGTTTGTTTGTAGAAAAACTAATTT 1292
                  ********  * ***** *********** *** ************    *  * ***

Rat             GGAACTATAAGAAAGACAGTGCACTGCCTGTAAATTCACAGTGTTTGGTAGAATCCTTTT 1408
Mouse           GGAACTATAAGAAAGACAGTGCACTGCTTGTAAATTCACAGTGTTTGGTAGAATCCTTTT 1407
Homo            G-AACTATAAGAAAGACAGTGCACTGCTTGTAAATTCACATTGTTTGGAAAAATTCTTTT 1351
                * ************************ ************ ******* * *** *****

Rat             GGAGCACACACACAAGAATAGGTACACAGCGACTGGTACCTTCTCTATTGTAAATATTTC 1468
Mouse           GGAGCACACACACA--AATAGGTACAAAGTGACTGGTACCTTATCTATTGTAAATATTTC 1465
Homo            GGAACAAAAA------ATTAGGTACATGATAACTGGTACCTTATCTACTGTAAATATTTC 1405
                *** ** * *       * ********     *********** **** ************

Rat             ATTCAAAATGATGCACATATAAATATATTCTTACAGAATTTGCTGTAATTGTTGATCCAT 1528
Mouse           ATTCAAAATGATGCACATATGAATATATTCTTACAGAATTTGCTGTAATTGCTGATCCAT 1525
Homo            ATTAAAAATGATGCACACATAGATATATTCTTACAAATTTTGCTGTA-TTGCTGTTCTCT 1464
                *** ************* **  ************* * ********* *** ** **  *

Rat             TTGAGGCTCTCCAAAGTTCTGAGTTCTGTATATGACCTCGTTCCTTGTTTTTGTTAATGA 1588
Mouse           TTGAGGCTCTCCAAAGTTCTGAGTTCTGTATATGACCTCGTTTCTTGTTTTTGTTAATGG 1585
Homo            TTGAGGCTCTCCAAAGTCTTGAGTTCTGTATATGGCCTGGTTTCTTGTTTTTATTAATAG 1524
                *****************  *************** *** *** ********* *****
```

Fig. 3.0: Alignment of the 3'UTR of Rat, Mouse and Human Brn-3b (Continued from previous page) The red boxes indicate the mir-128 and mir-330 binding sites respectively.

```
Rat             AT----TATTTACTATAGTAATGTATTAAGTTATTTTGGGTGTTGTTTCATTGTCTTTCA 1644
Mouse           GT----TATTTACTATAGTAATGTATTAAGTTATTTTGAGTGTTGTTTCATTGTCTTTCA 1641
Homo            ATGGTTTATTTACTATGGTAATGTATTAATTTATTTTTGGTGTTGTTCGATTGTCTTTCA 1584
                 *    ********** ************ *******  ********  ************

Rat             TCGAAGAGATGATTTTAATGTTTTATTGGCAAAGTATGCTACTTTTTTCATTAAAATATG 1704
Mouse           TTGAAGAGATGATTTTAATGTTTTACTGGCAAAGTATGCTGCTTTTTTCATTAAAATATG 1701
Homo            TTGAAGAGATAATTTTAATGTTTTATTGGCAACGTATGCTGCTTTTT-CATTAAAATATG 1643
                * ******** ************** ****** ******* ****** ************

Rat             CTATTAATATTAAATGACTTTTAAAACGTG 1734
Mouse           CTATTAATATTAAATGACTTTTAAAACGTG 1731
Homo            CTATTAAAATTAAATGGCTTTTA------- 1666
                ******* ******** ******
```

Fig. 3.0: Alignment of the 3'UTR of Rat, Mouse and Human Brn-3b (Continued from previous page)

The level of conservation of the 3'UTR of Brn-3b in different species suggests that this region could play an as yet unidentified role in its regulation. As reported in the introduction, post-transcriptional gene regulation can operate at the level of the 3'UTR of an mRNA (Fire et al. 1998; Kenealy et al. 2000; Pontrelli et al. 2004; Yang et al. 2005). The analysis of the 3'UTR of Brn-3b via bio-informatic tools (Targetscan.org and mirnaviewer at http://cbio.mskcc.org/mirnaviewer) showed the presence of putative microRNA binding sites which are evolutionary conserved (figure 3.0, red boxes). In order to understand whether these or other *in-cis* sequences contained in the 3'UTR of Brn-3b are involved in its regulation, this region was subcloned downstream of GFP in a reporter construct that would allow the detection and evaluation of this phenomenon.

3.1 Regulation of a reporter mRNA containing the 3'UTR of Brn-3b.

As reported in the introduction, Brn-3a and Brn-3b are oppositely regulated in ND7 cells induced to differentiate via serum removal (Smith et al. 1996). While Brn-3a

levels increase, Brn-3b levels are reduced (Smith et al. 1996). This phenomenon raised the question of whether serum removal in ND7 cells could induce the degradation of Brn-3b via mechanisms acting on its 3'UTR.

This region was thus sub-cloned downstream of GFP to obtain the peGFP::3bUTR construct as reported in section 2.17 of this thesis. The peGFP::3bUTR and the empty peGFP control constructs were transfected into ND7 neuroblastoma cells. After 24 hours, cells were induced to differentiate via serum removal and after 1, 3 or 5 days, the levels of GFP were assessed via western blot analysis (fig. 3.1). The levels of neomycin, expressed in the same vector, were used for normalization purposes.

As shown in figure 3.1 the levels of GFP are reduced in the GFP reporter construct containing the 3'UTR of Brn-3b compared to the vector expressing GFP alone.

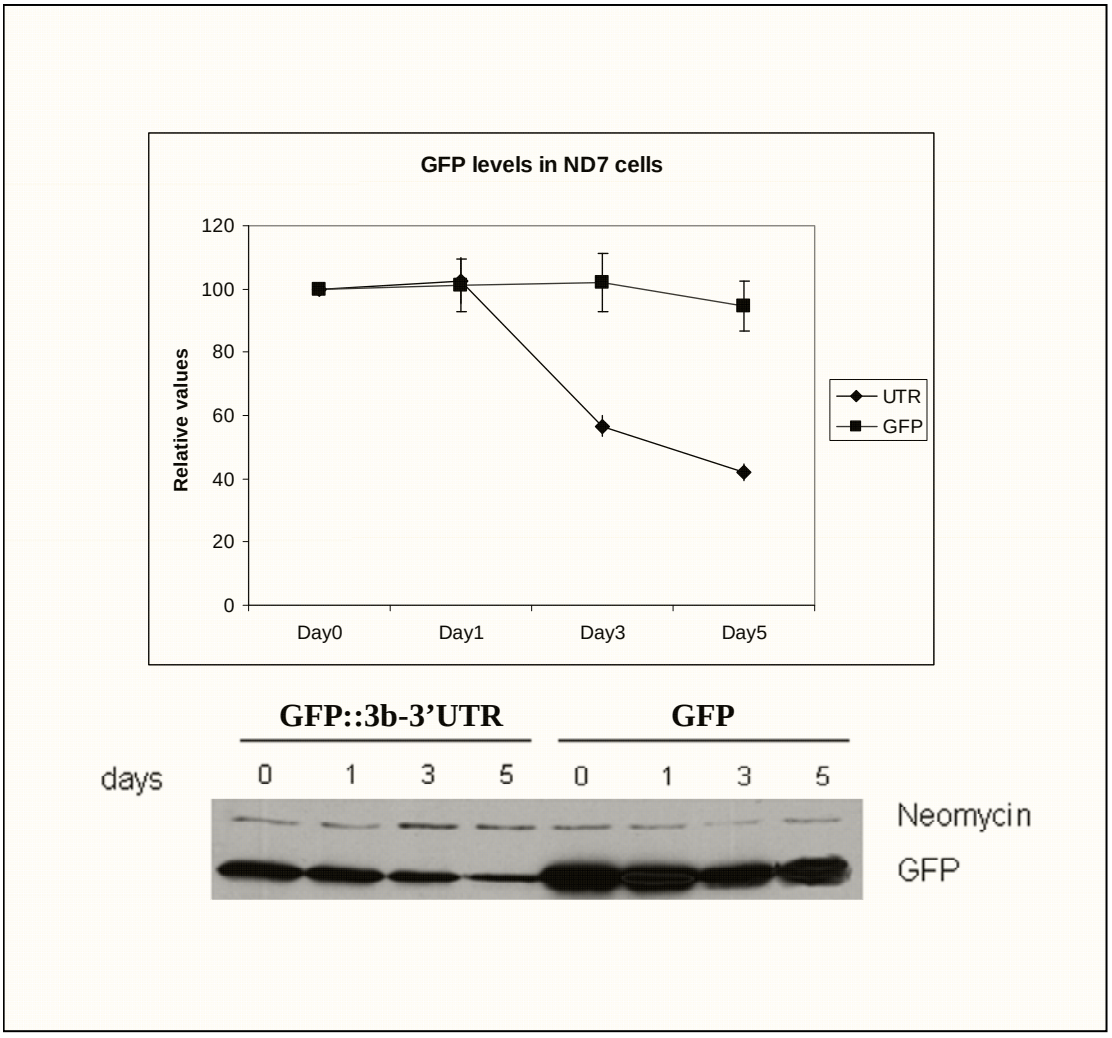

Fig. 3.1: Modulation of GFP levels following serum removal in ND7 cells. Downregulation of a reporter construct containing the 3'UTR of Brn-3b in ND7 cells induced to differentiate via serum removal. The graph represents the average of three experiments. The lower panel represents a typical western blot analysis (days=days without FCS). Bars indicate standard deviation.

In order to exclude that the reduction of GFP levels in cells transfected with the peGFP::3b-3'UTR construct could be due to increased cell death, the experiment shown in fig 3.1 was repeated by co-transfecting GFP::3b-3'UTR or GFP control vectors with a mCherry vector which expresses red fluorescent protein (RFP). If the reduced levels of GFP as seen when expressed in fusion with the 3'UTR of Brn-3b had toxic effect, the

levels of red fluorescence would have been reduced alongside the levels of green fluorescence compared to the control vector. As shown in fig 3.2 while the levels of green fluorescence are reduced in cells transfected with the GFP::3b-3'UTR and induced to differentiate via serum removal (panel D, GFP column), the amount of red fluorescence is the same in both the GFP::3b-3'UTR and GFP transfected cells and it is independent on serum removal (panel B, D, RFP column). The number of "green" and "red" cells were counted blind by another member of the laboratory and reported in a graph (fig 3.3) which shows that the loss of "green signal" is not associated to a reduction in the "red signal" when the 3'UTR of Brn-3b is expressed alongside GFP.

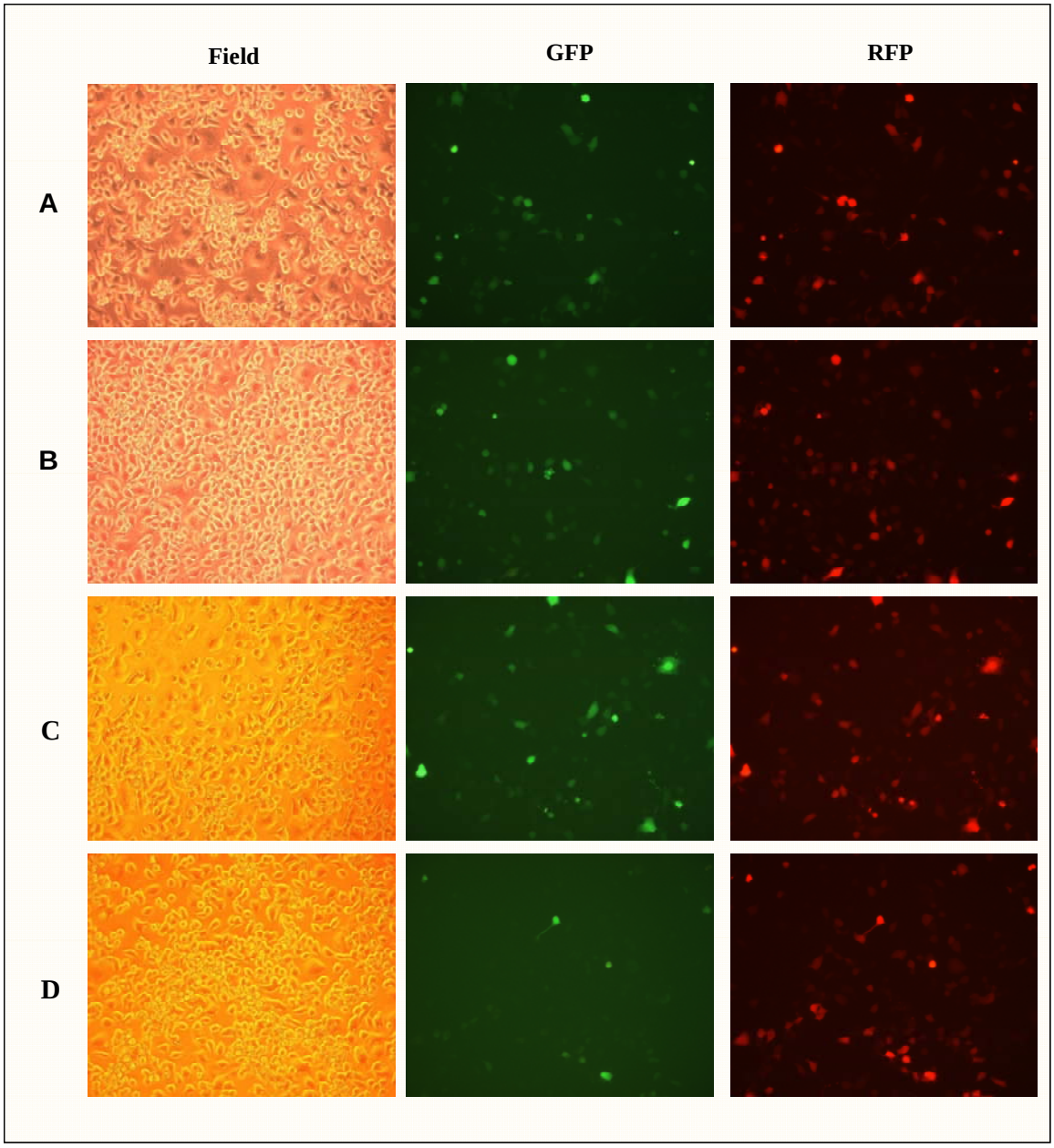

Fig. 3.2: Example of GFP and RFP expression in ND7 cells. (A-B): ND7 cells transfected with GFP and mCherry vectors and then incubated respectively with (A) or without serum (B) for two days.
(C-D): ND7 cells transfected with the peGFP::3b-3'UTR and mCherry and incubate respectively with (C) or without serum (D) for two days. GFP=green fluorescent protein, RFP=red fluorescence protein

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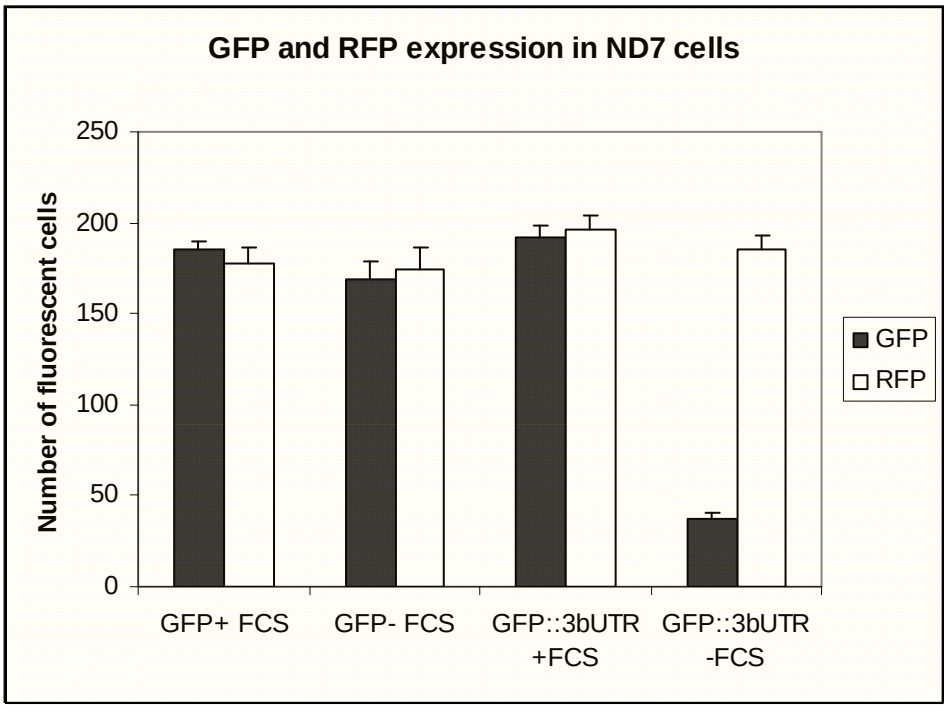


Fig. 3.3: Quantification of GFP and RFP expression in ND7 cells. Graphical representation of the average of three independent experiments measuring the number of fluorescent green and red cells in five different fields counted blind. The error bars indicate standard error.

This result suggests that the reduction of GFP levels in the presence of the 3'UTR of Brn-3b is likely to be due to a specific reduction of GFP-3b'3'UTR mRNA levels rather than increased cell death as mediated by the chimaeric construct.

In order to expand the result, the experiment reported in fig 3.1 was repeated in the SHSY-5Y human neuroblastoma and in the MCF-7 human breast cancer cell line, both of which express Brn-3b (Lee et al. 2005; Budhram-Mahadeo et al. 2008). This experiment was performed in order to investigate whether the down-regulation of Brn-3b

could be a neuronal-only phenomenon or whether it is independent of the origin of the cell type. As shown in figure 3.4, the levels of the chimaeric mRNA were reduced in the SH-SY5Y cell line but not in the MCF-7 cell line following serum removal. This suggests that the reporter mRNA containing the 3'UTR of Brn-3b is not intrinsically unstable. Although the experiment was performed in two neuronal cell lines and one breast cancer line only, this result prompted speculation on whether the degradation of the 3'UTR of Brn-3b occurred in neuronal cells only.

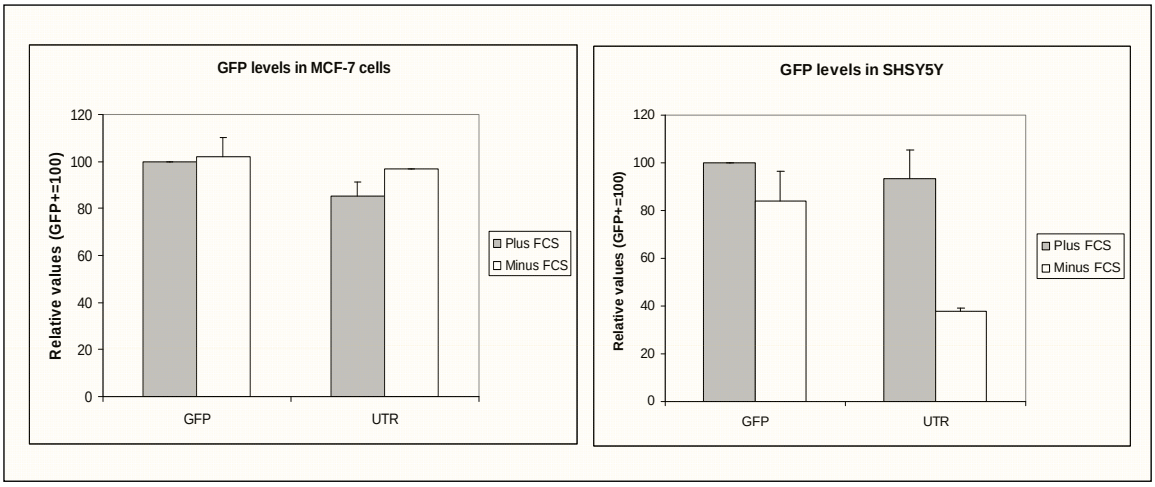


Fig. 3.4: Modulation of GFP levels in different cell lines. The SH-SY5Y and MCF-7 cell line have been transfected and treated as reported above and the levels of GFP evaluated by western blot. The graphs represent the average of at least three experiments and the error bar represents standard error.

3.2 Degradation of the mRNA containing the 3'UTR of Brn-3b

Since the GFP:3b-3'UTR and GFP vector differ only by the presence of the 3'UTR of Brn-3b in the former construct, it is unlikely that the observed reduction in

GFP protein levels is due to differential protein stability and suggested that the observed phenomenon occurred at the post-transcriptional level. In order to clarify this aspect, ND7 cells were transfected as reported above, serum starved and treated with Actinomycin-D. By inhibiting transcription, this compound allows the evaluation of mRNA turnover. Total RNA was extracted from ND7 cells treated with actinomycin-D and, following cDNA synthesis, subjected to realtime RT-PCR analysis with primers for GFP. As shown in figure 3.5, the levels of the mRNA containing the 3'UTR of Brn-3b are strongly and rapidly reduced compared to the control GFP mRNA. This indicates that the observed reduction of GFP at the protein level is due, at least in part, to the degradation of its mRNA when the 3'UTR of Brn-3b is present. Since both constructs are under the control of the CMV constitutive promoter, no differential transcriptional regulation is likely to occur, and post-transcriptional regulation acting on sequences contained within the 3'UTR of Brn-3b is therefore likely to be responsible for reduced GFP levels.

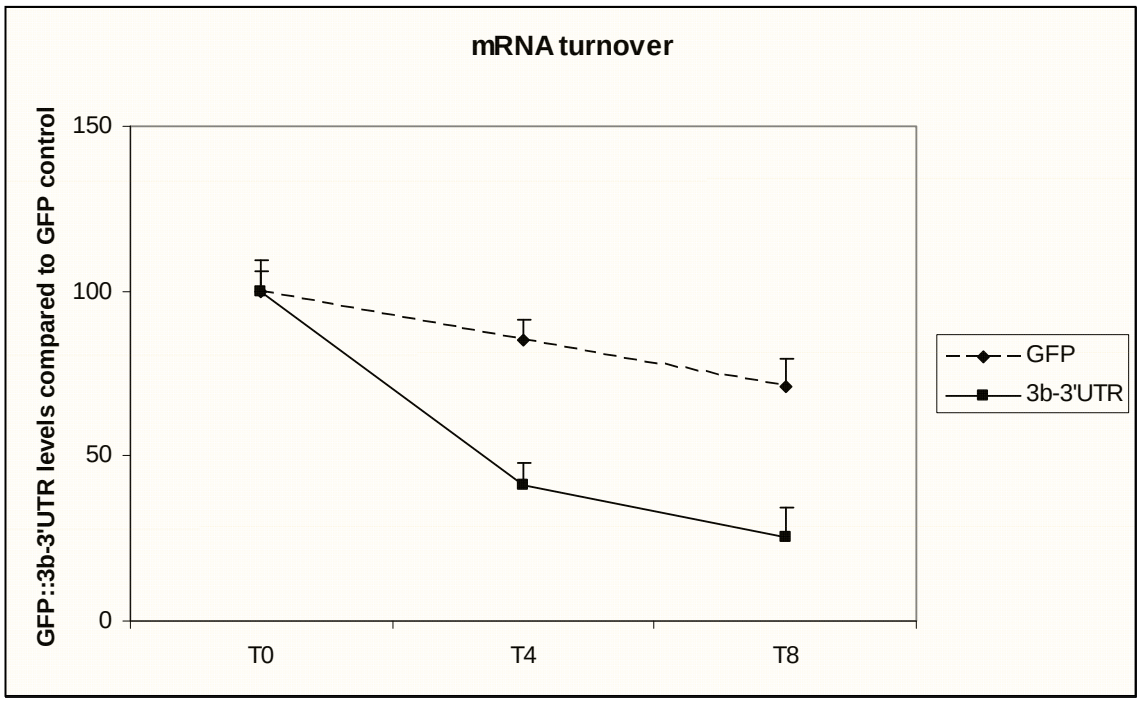


Fig. 3.5: GFP mRNA turnover analysis. ND7 cells were transfected with the indicated constructs and subsequently treated with Actinomycin D for the indicated hours. Total RNA was then extracted and its cDNA used as a template in a realtime qRT-PCR reaction with primers specific for GFP. Values were normalized versus the levels of neomycin which is expressed from the same construct. Error bars indicate standard error.

3.3 Sequential deletion of the 3'UTR of Brn-3b with *Bal-31*

In order to map the region of the 3'UTR of Brn-3b responsible for this effect, a series of deletion constructs were made in this region with the *Bal-31* exonuclease and subsequently assessed for their sensitivity to differentiation-induced degradation using the assay described above. *Bal-31* (as reported in figure 3.6) is an exonuclease enzyme which degrades both 3' and 5' ends of duplex linear DNA without causing any internal excision.

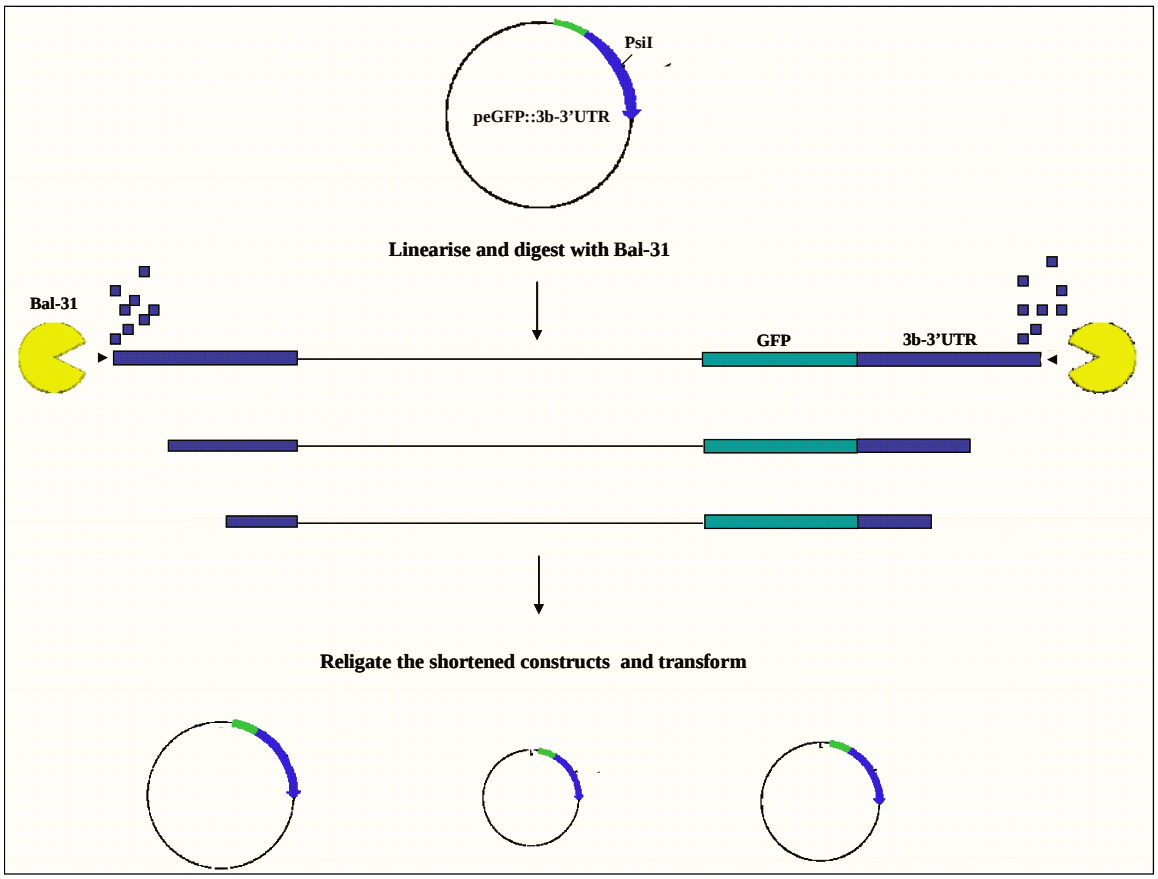


Fig. 3.6: Mechanism of *Bal-31* activity. *Bal-31* digests duplex linear DNA from both its 3' and 5' termini allowing a time-controlled shortening of DNA. The figure schematically represents the strategy adopted in order to reduce the length of the 3'UTR of Brn-3b.

The digestion rate and hence the resulting length of the DNA fragment can be regulated by controlling the incubation time of *Bal-31* (figure 3.6). In order to evaluate the nuclease activity of *Bal-31* in this system, a test deletion was initially performed on the peGFP::3bUTR, linearized with the *PsiI* restriction enzyme. This enzyme cuts the 3'UTR of mouse Brn-3b in the middle and the linearized vector has thus half of this region at

each end. Digested products were run on an agarose gel for visualization and evaluation purposes. The distance of the DNA bands from the well in which they were loaded was plotted on a semi-logarithmic scale versus the marker represented at the left, as shown in the inset in figure 3.7. This test digestion allowed to evaluate the relationship between *Bal-31* activity versus time on the peGFP::3b-3'UTR construct and to calculate that the digestion rate is approximately 75 base pairs (bp) of DNA per minute.

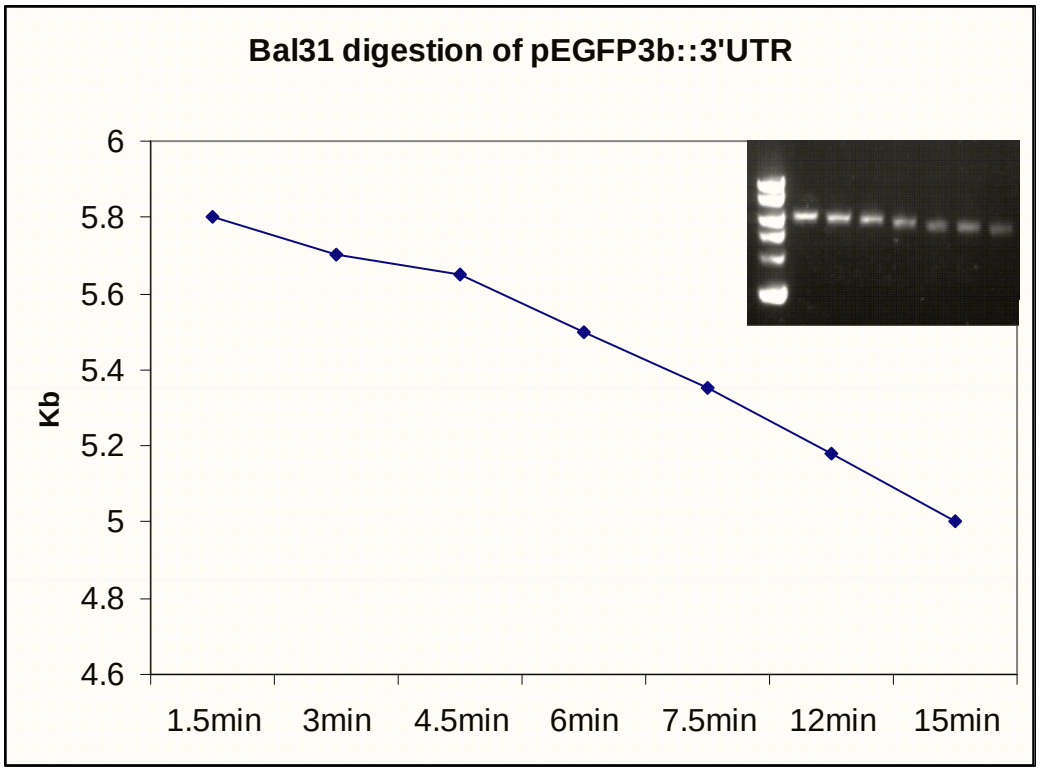


Fig.3.7: Time-dependent activity of *Bal-31*. The peGFP::3b-3'UTR construct was linearized with the *PsiI* restriction enzyme, incubated with *Bal-31* for the times indicated and run on an agarose gel (inset).

Subsequently a reaction was set up consisting of a linearized peGFP::3b'3UTR construct and *Bal-31* in the appropriate buffer and aliquoted in several tubes kept on ice. All tubes

were then transferred to *Bal-31* operating temperature. At different time points the reaction was stopped, the construct re-ligated and transformed into competent bacteria. Minipreparations of DNA were subjected to a restriction enzyme digest to release the insert and to measure its length as shown in figure 3.8.

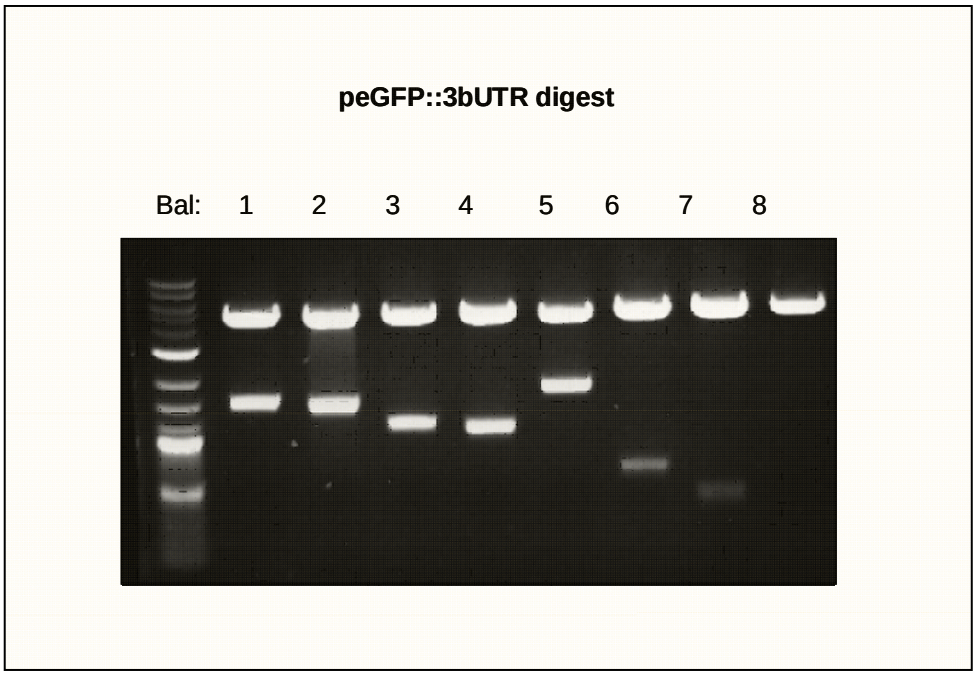


Fig 3.8: 3b-3'UTR insert release from *Bal-31* treated peGFP::3b-3'UTR construct. The peGFP::3b-3'UTR construct was linearized with *PsiI*, incubated with *Bal-31* for the time reported in figure 3.6, re-ligated and transformed in competent DH5α E.coli cells. A DNA minipreparation from bacterial colonies was subjected to a digestion with *SacI* and *BamHI* restriction enzymes to release the insert.

With this approach several *Bal-31* deletion mutants ranging from ~1.6 Kb (Bal1) to ~500bp (Bal7) were obtained. From Bal8 onwards, no insert was left and the constructs were thus not used for any subsequent experiment. Due to the fact that Bal1 and Bal2 as well as Bal 3 and Bal4 are similar to one another, only the Bal1, Bal4, Bal6 and Bal7

deletion mutants were subsequently used in GFP assay to evaluate their responsiveness to serum removal (Fig.3.9). The reaction in lane 5 seems not to have been digested by Bal-31 and the insert released following digestion with SacI and BamHI is the size of wild type 3b::3'UTR.

3.4 Analysis of *Bal-31* deletion mutants.

The four *Bal-31* deletion mutants were then transfected in ND7 cells in order to assess whether the responsiveness to serum removal had been diminished. As shown in the figure below (figure 3.9), while the Bal1 deletion mutant still responds to serum removal like the wild type (3b-3'UTR), the Bal4, Bal6 and Bal7 mutants have lost this responsiveness, suggesting that the *in–cis* sequences responsible for the down-regulation of the chimaeric mRNA harbouring the 3'UTR of Brn-3b have been removed.

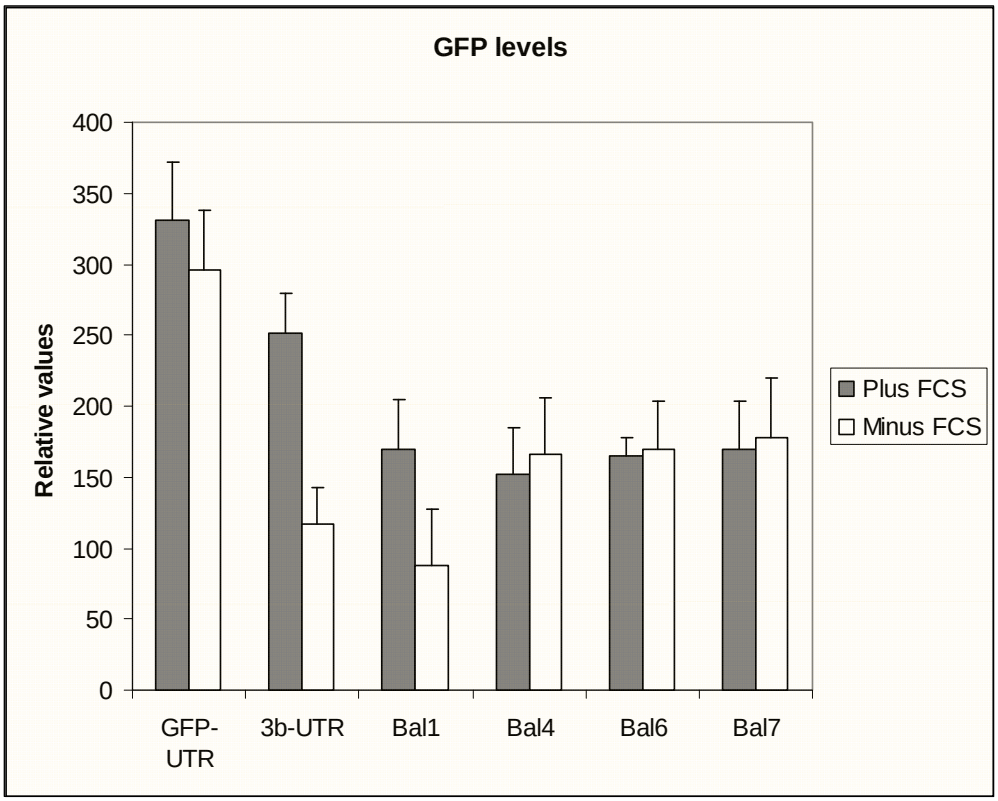


Fig. 3.9: Analysis of GFP levels of the peGFP3b::3'UTR deletion mutants. The *Bal-31* deletion mutants described in figure 3.7 were transfected in ND7 cells alongside their wild type counterpart and a GFP vector in an assay directed to measure their responsiveness to serum removal.

All the Bal-31 deletion mutants were sequenced and analysed using bioinformatics tools for known sequences that might influence mRNA stability at the post-transcriptional level (Jacobs et al. 2006) but, apart from microRNA binding sites as shown in figure 3.0, no AU-rich or other known sequences were found.

The Bal1 mutant responds to serum removal as the wild type and it is likely therefore that the deleted region containing the mir-128a/b binding site is not involved in

the serum deprival mediated degradation of the reporter construct. In contrary, Bal4, Bal6 and Bal7 mutants, have lost the responsiveness to serum removal and their deleted region is putatively targeted by three different microRNAs: mir-23, mir-128, and mir-214, one of which, mir-23, has two target sites on the 3'UTR of Brn-3b (figure 3.10).

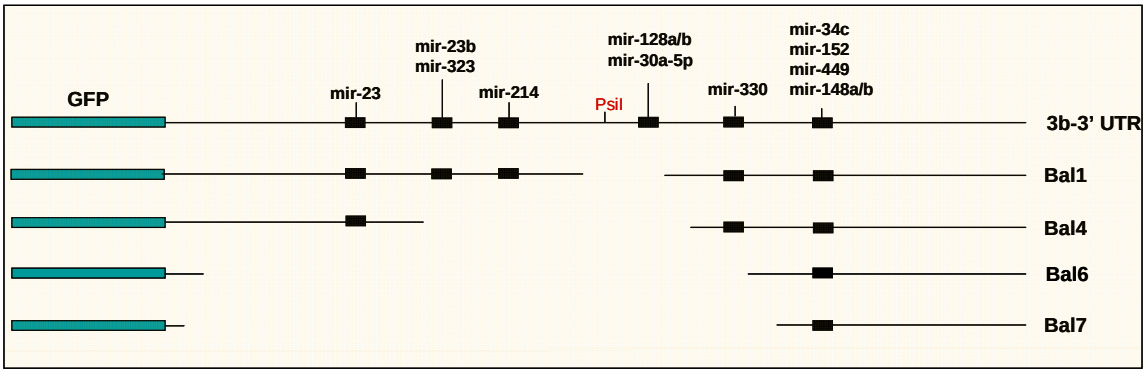


Fig. 3.10: MicroRNA target site localization on the 3'UTR of Brn-3b. Schematic representation of the indicated microRNA putative binding sites on the wild type 3'UTR of Brn-3b and on its *Bal-31* deletion mutants. The *PsiI* restriction site is in red typeface.

These microRNAs putatively bind to highly conserved (90-100%) sites in the 3'UTRs of mouse and human Brn-3b and this evolutionary conservation further supports a possible regulatory role (figure 3.10 and table 3.0). Furthermore none of these putative microRNAs have any matching binding sites on the 3'UTR of the closely related Brn-3a transcription factor which is up-regulated doing ND7 cell differentiation suggesting specificity for Brn-3b (data not shown).

	Mouse/human conservation	microRNA overall homology on mouse Brn-3b 3'UTR.	microRNA "seed" homology on mouse Brn-3b 3'UTR.
Mir-23 (upstream)	95 (19/20)	65 (13/20)	100 (7/7)
Mir-23 (downstream)	100 (20/20)	80 (16/20)	100 (7/7)
Mir-128	90 (18/20)	70 (14/20)	71 (5/7)
Mir-214	90 (18/20)	65 (13/20)	100 (7/7)

Table 3.0: Putative microRNA target sites in the 3'UTR of Brn-3band their conservation.

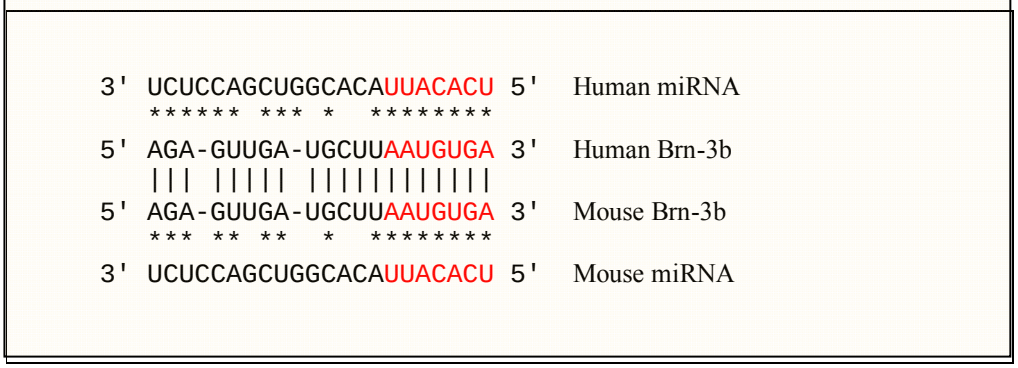
3.5 Mutation of the microRNA binding sites on the 3'UTR of Brn-3b

In order to determine whether these microRNAs play a specific role in the degradation of the GFP::3b-3'UTR reporter construct, their putative target sequences on the 3'UTR of Brn-3b were individually mutagenised by changing three out of the seven nucleotides, known as the "seed" (shown in red in table 3.1) which is essential for the binding of the microRNA to its target sequence.

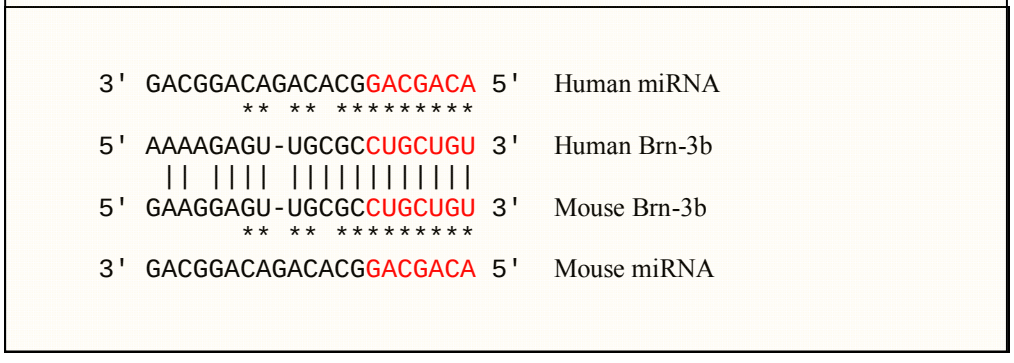
Mir-23 (upstream)
Seed conservation: 100%

```
3' CCUUUAGGGACCGUUACACU 5'   Human miRNA
     *** ** *  *******
5' AACAAUUCCAGUAAAUGUGA 3'   Human Brn-3b
   ||||||||| ||||||||||
5' AACAAUUCCGGUAAAUGUGA 3'   Mouse Brn-3b
     *** ** *  *******
3' CCUUUAGGGACCGUUACACU 5'   Mouse miRNA
```

Mir-23 (downstream)
Seed conservation: 100%



Mir-214
Seed conservation: 100%



Mir-128
Seed conservation: 71%

```
3' UUUUCUCUGG-CCAAGUGACACU 5'   Human miRNA
       ***** * ********
5' CGUGUAGACCUGAUUCACUGUCU 3'   Human Brn-3b
   |||||||||| ||||||||||
5' CGUGUAGACCAGAUUCACUGUCC 3'   Mouse Brn-3b
       ***** * ********
3' UUUUCUCUGG-CCAAGUGACACU 5'   Mouse miRNA
```

Table 3.1: Putative microRNA binding sites on the 3'UTR of Brn-3b. Each box represents the evolutionary conservation of the microRNA binding sites and related microRNA in the 3'UTR of murine and human Brn-3b. The identities between miRNA and 3'UTR are indicated by asterisks. The seven nucleotide region known as the "seed" is highlighted in red letters.

The GFP levels resulting from the mutated constructs were assessed by western blot. As shown in figure 3.11, the mutagenesis of the sites targeted by mir-23 (A, B), or mir-214 (C) but not by mir-128 (D) seems sufficient to avoid the reduction of GFP following serum removal. This result is consistent with the observation reported in section 3.4 (and also figure 3.9 and 3.10) that the Bal1 mutant, which lacks the mir-128 binding site, still responds to serum removal, while Bal4 and Bal7 mutants, lacking the mir-23 and mir-214 binding sites no longer have this response.

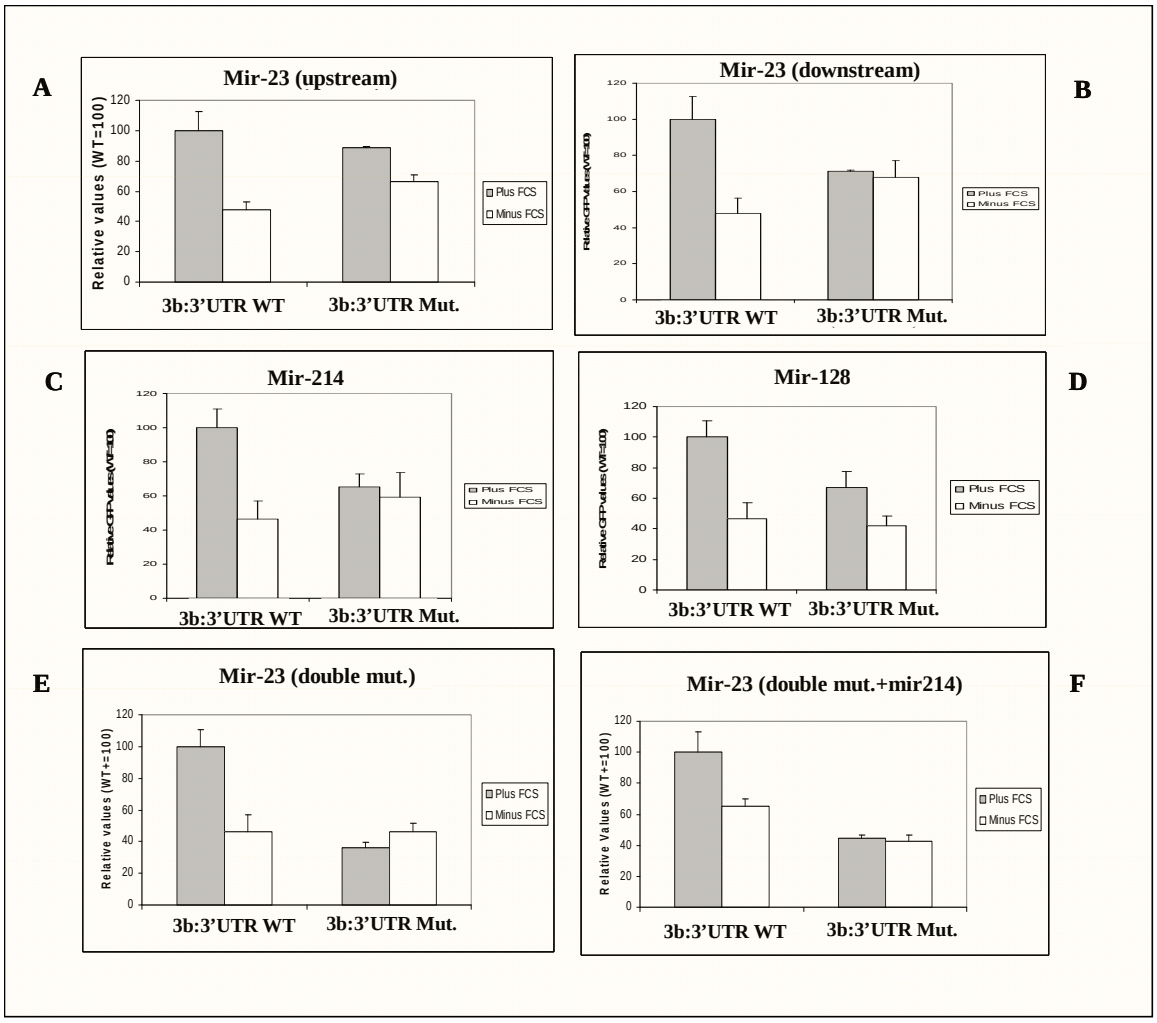


Fig. 3.11: Effect of mutations of microRNA binding sites. The indicated microRNA binding sites on the 3'UTR of Brn-3b were individually mutagenised and their role measured as the amount of GFP protein produced by each construct with or without serum as measured via western blots. The scale is the same for all graphs and it has been normalized to 3b-3'UTR WT plus FCS=100.

The 'downstream' mir-23 target site (B) seems more important than the 'upstream' one (A) as the mutagenesis of this binding site produces a stronger reduced response to serum removal. This is also reflected by the level of homology of this region in mouse and human since the 'downstream' mir-23 binding site on the 3'UTR of Brn-3b is more

conserved than the 'upstream' one. In contrast, mir-128, which in this assay does not seem to have any role in the regulation of the chimaeric construct, has a very low homology with its target site on the Brn-3b 3'UTR, especially at the critical 5' "seed" end of the microRNA (table 3.1, last panel).

Intriguingly, the introduction of mutations in the microRNA binding sites seems to trigger a reduced production of GFP in the presence of serum. This is particularly evident in the mir23/mir23 double mutant (E), in which both mir-23 sites have been mutagenised and in the mir23/mir23/mir214 (F) triple mutant in which the mir-214 site has also been mutagenised. In both instances the overall levels of GFP are considerably lower than mutants harbouring mutations only in one microRNA binding site. The analysis of this phenomenon is outside the scope of this thesis but it is possible that cells are able to identify heavily mutated and thus potentially "dangerous" mRNAs and dispose of them accordingly.

3.6 Effect of the chimaeric constructs on the expression of endogenous Brn-3a and Brn-3b.

The analysis of the regulation of the reporter construct containing the 3'UTR of Brn-3b triggered questions regarding the fate of the endogenous Brn-3b in similar experimental circumstances. The next step was thus to understand whether a similar phenomenon occurs to the endogenous Brn-3b mRNA.

ND7 cells were transfected with the peGFP::3bUTR or GFP control vector and the levels of the endogenous Brn-3a and Brn-3b mRNA evaluated following serum removal as described previously. If specific microRNAs are directly involved in the

downregulation of Brn-3b, then the transfection of a reporter construct containing the 3'UTR of Brn-3b should "titrate" all the available microRNAs and reduce or block endogenous Brn-3b degradation. Given the lack of any significant homology between the 3'UTR of Brn-3a and Brn-3b, the same treatment should have no effect on the levels of Brn-3a. As shown in figure 3.12, the endogenous Brn-3b mRNA levels are not reduced following serum removal in ND7 cells transfected with the peGFP::3bUTR, but are reduced as normal in the presence of the 'empty' GFP vector. In the same experimental conditions, the levels of the endogenous Brn-3a mRNA increase during serum removal as expected.

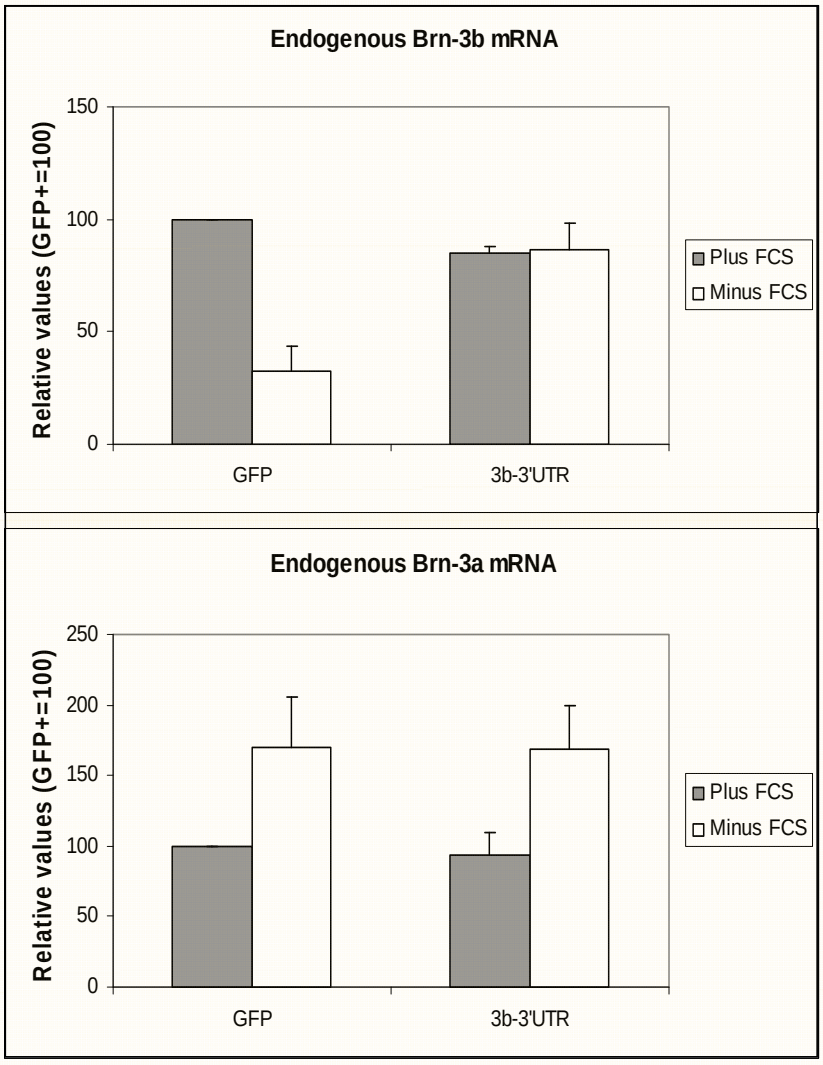


Fig 3.12: Expression levels of the Brn-3a and Brn-3b endogenous mRNA. ND7 cells were transfected with the peGFP::3b-3'UTR or empty GFP vector and induced to differentiate via serum removal. Total RNA was extracted and used for a qPCR using specific primers. The graph represents the average of at least three experiments.

3.7 Expression of microRNAs and Dicer in ND7 cells

Following the identification of specific microRNAs as possibly involved in the modulation of Brn-3b, it became important to evaluate their expression levels in ND7 cells grown in full medium or without serum. A realtime RT-PCR approach was initially

used to evaluate the levels of specific pri-miRNA. Unfortunately, a high level of non-specific products and high variability in the obtained results (data not shown) led to discard this technique in favour of a northern blot analysis. Contrary to a PCR-based system in which small variations of the amount of the original template can result in big differences at the end of the exponential amplification reaction, a northern blot technique offers the advantage of giving a linear representation of the amount of specific microRNAs (or mRNAs) and giving thus a potentially more realistic result than a PCR-based approach.

Total RNA extracted from ND7 cells grown with or without FCS, was thus subjected to northern blot analysis with mir-23, mir-214, mir-128, mir-96 and mir-152 probes. The first three microRNAs have been chosen because they have a putative target site on the 3'UTR of Brn-3b, with mir-23 and mir-214 putatively having a role in its degradation while mir-128 apparently without any role in the modulation of Brn-3b. Mir-96 and mir-152, instead, although expressed in mouse, do not have a putative target site on the 3'UTR of Brn-3b and are thus used as controls to establish whether the potential modulation of microRNA expression is a general or specific for some microRNAs. Mir-96 is predominantly expressed in the olfactory epithelium (Bak et al. 2008) and mir-152 predominantly expressed in the pituitary gland (Bak et al. 2008) of mice. Both control microRNAs are thus expressed in mouse nervous system (Hohjoh et al. 2007; Bak et al. 2008). As shown in figure 3.13, mir-23 and mir-214, are detectable and their levels are increased in ND7 cells subjected to serum removal. Mir-128 is barely detectable and its expression is not changed by serum removal. Mir-96 and mir-152 are detectable in ND7 cells but their levels are not modified following serum removal. This result suggests that

mir-23 and mir-214 are specifically up-regulated in ND7 cells following their differentiation induced by serum removal.

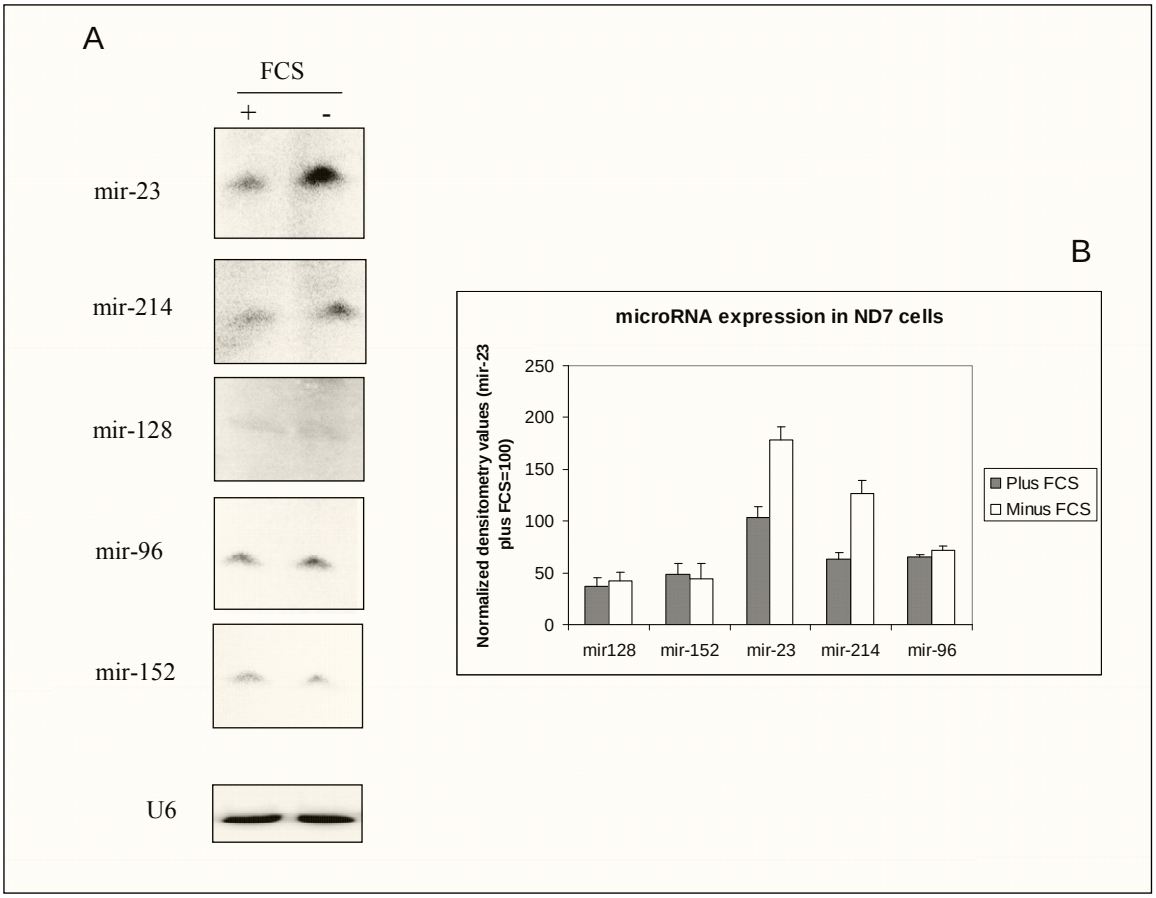


Fig 3.13: Northern blot analysis of microRNAs expression in ND7 cells. Total RNA was extracted from ND7 cells grown with or without FCS and used for a northern blot analysis (see material and methods for an extended protocol). U6 was used to normalize RNA loading. (A), typical northern blot for the indicated microRNAs and (B), graphical representation of the effect of serum removal on the levels of the indicated microRNAs as evaluated by densitometry analysis of three independent northern blots.

The mRNA and protein levels of Dicer, one of the key enzymes involved in the production of mature microRNAs, were next evaluated in order to understand if it was regulated in ND7 cells following serum removal. As shown in figure 3.14, Dicer is expressed in ND7 cells and intriguingly both its mRNA and protein levels seem to be up-

regulated following serum removal. Due to the key role played by Dicer in the production of microRNAs, this result prompted speculation regarding the fate of microRNAs in ND7 cells. It would have been extremely interesting, for instance, to assess whether the reduction in serum levels in ND7 cells would be a trigger of a global increase of microRNAs but due to time constraints and in order to keep the focus of the experimental work on the regulation of Bnr-3b by microRNAs, the mechanisms and the effect of the manipulation of Dicer levels, will be investigated in future work. Nonetheless, the lack of mir-96 and mir-152 increase in ND7 cells growing without serum, seem to suggest that, although Dicer levels might increase, this is not necessarily associated to an increase in microRNA levels.

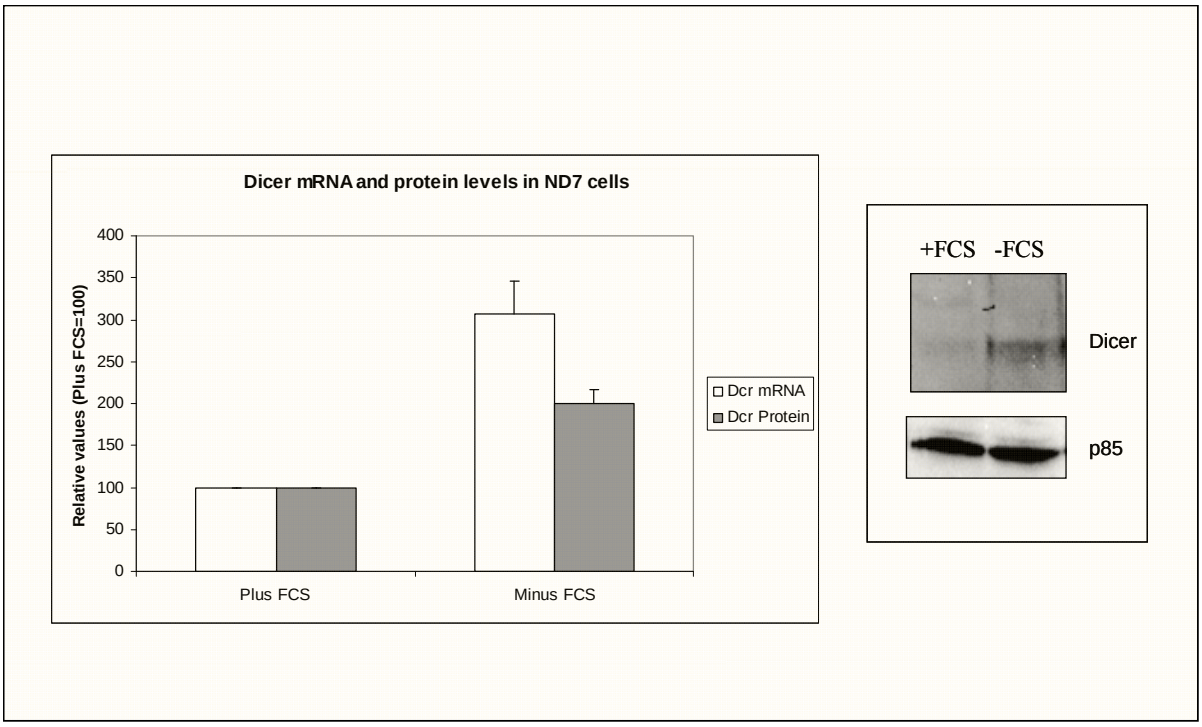


Fig 3.14: Dicer expression levels in ND7 cells. Total proteins were extracted from ND7 cells induced to differentiate via serum removal or kept in full serum and subjected to a western blot analysis with an anti-Dicer antibody. The blots were normalized with p85. The graph on the left represents the average of at least three experiments.

3.8 Discussion

The Brn-3b transcription factor, a POU IV-class protein, is modulated in retinal ganglion cells (RGCs) during development (Xiang et al. 1993; Xiang 1998). Furthermore its expression levels are reduced upon differentiation of the ND7 cell line (Smith et al. 1996). Intriguingly, the 3'UTR, a region well-known to be a target for post-transcriptional regulation (Kenealy et al. 2000; Andreassi et al. 2009), is highly conserved amongst Brn-3b in different species. By using different approaches, it is suggested that the 3'UTR of Brn-3b is directly involved in the degradation of a reporter chimaeric mRNA putatively via microRNAs binding specific sequences. This effect occurs in the neuronal ND7 and SHSY-5Y but not in the MCF-7 breast cancer cell line. Brn-3b is normally expressed in all these cell lines (Lee et al. 2005; Budhram-Mahadeo et al. 2008). Although very few cell lines have been used to draw any conclusions, as a working hypothesis the data presented so far seemed to suggest that the microRNA-mediated degradation of Brn-3b could be a phenomenon that occurs only in neuronal cells. This working hypothesis will be analyzed and disproven in the next chapter when more data had become available.

The initial data concerning the fate of the GFP chimaeric mRNA harbouring the 3'UTR of Brn-3b, had left open the question on whether the observed down-regulation was due to degradation of the mRNA or vicerversa simply a consequence of increased cell death due to some intrinsic toxicity of the peGFP::3b-3'UTR constructs compared to the control GFP. The latter hypothesis was excluded by showing that cells transfected with the peGFP::3b-3'UTR or control GFP vector alongside a vector encoding for a red fluorescent protein and induced to differentiate via serum removal showed a reduced

level of GFP in the presence of the 3'UTR of Brn-3b but no differences of red fluorescence across all samples. If increased cell death was the cause of the reduction in GFP levels in the presence of the 3'UTR of Brn-3b (fig.3.1 and fig.3.2), then a comparable reduction in red fluorescence would have been observed. Furthermore, the expression levels of neomycin, expressed from within the peGFP vector, do not decrease in cells transfected with peGFP::3b-3'UTR compared to cells transfected with control GFP vector suggesting that the construct is not associated to any increased cell death (fig 3.1) following differentiation induced by serum removal.

The experiment with actinomycin D aimed at evaluating the degradation rate of the chimaeric mRNA containing the 3'UTR of Brn-3b in comparison to control GFP, seems to indicate that the 3'UTR of Brn-3b mediates an increased rate of the reporter mRNA harbouring it compared to the control. This evidence is in accord with the proposed mechanism of action of microRNAs, whereby a perfect match between a microRNA and its target site leads to the degradation of the mRNA. As shown in table 3.0, the mir-23 and mir-214 have perfect complementary matches on their target sites on the 3'UTR of Brn-3b.

The individual mutagenesis of candidate microRNA binding sites in the 3'UTR of Brn-3b has shown that mir-23 and mir-214 but not mir-128 are likely to be involved in the down-regulation of Brn-3b upon serum removal in ND7 cells. Two other microRNAs, mir-96 and mir-152, both expressed in mouse (Hohjoh et al. 2007; Bak et al. 2008) did not show any modification of their expression levels following serum removal. This seems to suggest that the modulation of mir-23 and mir-214 levels could have a specific role regarding the differentiation of ND7 cells. It would be intriguing to perform a

microarray analysis on microRNAs extracted from ND7 cells induced to differentiate via serum removal compared to untreated ones and analyse the modification of microRNAs involved in cellular differentiation. Furthermore, the mRNA and protein levels of Dicer, one of the two enzymes involved in the production of mature microRNAs (Bernstein et al. 2001; Lee et al. 2003), are also up-regulated in ND7 cells subjected to differentiation. The up-regulation of Dicer levels could have a global effect on the levels of microRNAs and it would thus be intriguing to evaluate this aspect in future work. Furthermore, given the role played by Drosha in the microRNA pathway (Lee et al. 2003; Carmell et al. 2004), it would be interesting to evaluate whether its expression is also subjected to a similar regulation as Dicer in ND7 cells induced to differentiate via serum removal.

The 'downstream' mir-23 binding site on the 3'UTR of Brn-3b is part of a consensus sequence termed 'K-box' (cUGUGAUa) (Lai et al. 1998). This was originally identified in the 3'UTR of *Notch* pathway target genes such as the ones belonging to *Enhancer of split* complex (Lai et al. 1998; Lai 2002). The K-box present in the 3'UTR of *E(spl)m8,* one of the members of the *Enhancer of split* complex (Lai et al. 1998), is sufficient to mediate the post-transcriptional regulation of a reporter construct harbouring it (Lai et al. 1998). It has been reported that *Notch* pathway is involved in the differentiation of RGC during development (Silva et al. 2003), whereby the reduction of *Notch* expression increases retinal ganglion cell genesis (Silva et al. 2003). The down-regulation of the *Notch* pathway (Nelson et al. 2006) leads to the up-regulation in RGCs of Brn-3b (Wang et al. 2001) and Math5 (Silva et al. 2003), another transcription factor involved in RGC differentiation. Due to presence of the K-box motif on the 3'UTR of

Brn-3b (Lai et al. 2005) it would be intriguing to speculate that the "downstream" mir-23 putative site on the 3'UTR of Brn-3b is thus a target of *Notch* signaling pathway (Lai 2002; Kawasaki H. 2005; Lai et al. 2005).

The role of the 3'UTR of Brn-3b in the degradation of the chimaeric GFP mRNA has also been indirectly confirmed by evaluating its effect on the modulation of the endogenous Brn-3b transcription factor. In comparison to cells transfected with the GFP control vector, the endogenous Brn-3b mRNA is not down-regulated in cells transfected with the pGFP::3bUTR reporter construct. A possible explanation of this phenomenon concerns the "titration" of specific microRNAs involved in the down-regulation of Brn-3b by the 3'UTR of the exogenous construct. This is also confirmed by the lack of any effect on the closely related Brn-3a transcription factor which is known to be up-regulated in differentiating ND7 cells. Since the 3'UTR of Brn-3a and Brn-3b do not have any similarity, these data confirm the specificity of the observed effect as mediated by the 3'UTR of Brn-3b. This effect could also be used experimentally to block the down-regulation of Brn-3b or any other mRNAs which are modulated via their 3'UTR and control their levels *in vitro* at a post-transcriptional level.

Although the mutagenesis of the microRNA binding sites on the 3'UTR of Brn-3b seems sufficient to alleviate serum starvation-induced down-regulation of GFP::3bUTR, these mutants display overall lower GFP levels compared to the 'wild type' GFP vector. The data presented in this thesis suggest that, as well as affecting the degradation of the GFP::3bUTR mRNA as mediated by microRNAs, the mutagenesis of the 3'UTR of Brn-3b seems to be causing *per se* an increased degradation. This is especially evident in the

mir-23/mir-23 double mutant (Fig.3.11 E) or in the mir-23/mir-23/mir-214 triple mutant (Fig.3.11 F) in which the overall levels of GFP, instead of increasing due to the elimination of progressively more microRNA binding sites, are instead lower than their wild type counterpart. These data suggest that the down-regulation of the different mutant GFP::3bUTR constructs is a process which possibly involves two separate mechanisms. One, mediated by microRNAs, seems to be involved in the specific down-regulation of the GFP::3bUTR mRNA as triggered by serum removal; the other instead seems to be causing a microRNA- and differentiation stimuli-independent degradation of the chimaeric mRNA. Although at the present time the mechanism involved in the latter phenomenon is not clear, it is possible to speculate that, as described previously, the chimaeric mRNA harbouring a mutated 3'UTR has an altered secondary structure and is therefore degraded (Monde et al. 2000; Puga et al. 2005).

MicroRNAs can potentially bind several target sequences (Burgler et al. 2005; Rusinov et al. 2005). Based on data available at the moment of writing this thesis and retrieved from a public available database at http://targetscan.org/, mir-23 and mir-214 can theoretically bind 672 and 348 target different mRNAs respectively. Intriguingly, beside Brn-3b, only 34 mRNAs of both lists contain target sequences putatively bound by both mir-23 and mir-214 (data not shown). Mir-96 and mir-152 (the control microRNAs used in figure 3.12), can putatively target 715 and 417 different mRNAs respectively. The number of mRNAs targeted by both microRNAs is 17. Although these in-silico data provide little information regarding the possible physiological effect of such joint regulation, it is possible that future experiments and advancements in computing will

help scientists to elucidate whether the multiple binding of different microRNAs on one target has a specific role in the regulation of mRNAs.

The analysis of microRNA expression levels has shown that mir-23, mir-214 and mir-128 are expressed in ND7 cells and that mir-23 and mir-214 are up-regulated in ND7 cells subjected to serum removal. In order to further confirm their specific role on the modulation of Brn-3b mRNA, it would be intriguing to reduce or silence the expression of endogenous mir-23 and mir-214 by specific siRNA and assess whether this reduction has any effect on the levels of endogenous Brn-3b in ND7 cells subjected to differentiation stimuli.

The next chapter will focus on analysing how the 3'UTR of Brn-3b is regulated in the RGC-5 retinal ganglion cell line which constitutes a more physiological system for Brn-3b.

Chapter 4

Modulation of Brn-3b in the RGC-5 cell line

4.0 Regulation of Brn-3b 3'UTR in RGC-5 cells

To test whether the 3'UTR of Brn-3b is subjected to post-transcriptional regulation in the RGC-5 cell line, the GFP::3b-3'UTR reporter construct was used and the levels of GFP measured by a western blot analysis. RGC-5 and, as a control, ND7 cells, were transfected with the aforementioned construct or a GFP vector as control. It has been previously reported in this thesis that the GFP::3b-3'UTR mRNA is down-regulated upon serum removal in ND7 cells, a treatment known to induce differentiation of ND7 cells and an upregulation of mir-23 and mir-214 expression. In order to achieve a similar response, transfected RGC-5 cells were induced to differentiate using staurosporine (SS) or depolarize using KCl rather than with serum removal which has no effect on this cell line (Harvey et al. 2007). In RGC-5 cells staurosporine induces the expression of Thy-1, a typical RGC differentiation marker (Lieven et al. 2007). KCl induces calcium influx in RGC-5 cells which induces the redistribution of protein complexes in this cell line (Selamat et al. 2009). This treatment could thus have an effect on the activity of the proteins involved in the miRNA silencing pathway. As a control, transfected cells were left in full serum.

Although the evidences described in chapter 3 concerning the regulation of the GFP::3b-3'UTR, were based on few cell lines only, the experiments seemed to suggest a working hypothesis by which the GFP::3b-3'UTR construct could be down-regulated in neuronal cells only. Surprisingly, the GFP reporter construct harbouring the 3'UTR of Brn-3b is not down-regulated in RGC-5 cells maintained in full serum. Furthermore, neither treatment induces any further down-regulation of the GFP levels (figure 4.0, panel

A). In comparison, and as described previously in this thesis, the same construct is down-regulated in ND7 cells induced to differentiate via serum removal (figure.4.0, panel B).

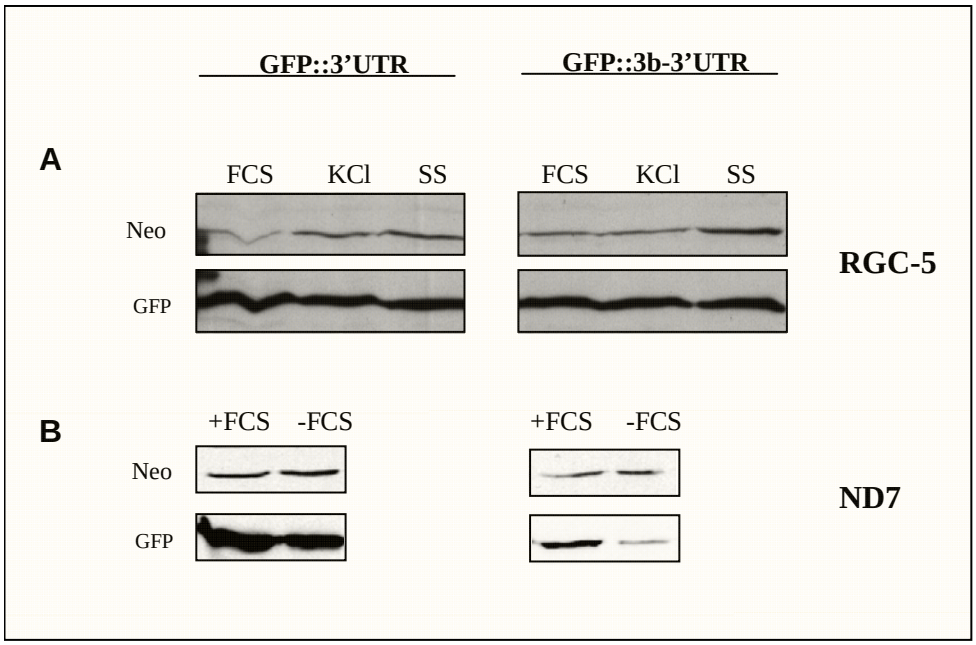


Fig. 4.0: Western blot analysis of GFP levels in RGC-5. RGC-5 cells were transfected with the indicated construct and subsequently treated as reported above and subjected to a western blot analysis with an anti-GFP antibody. The experiment was normalized with anti-neomycin antibody for loading differences. SS=Staurosporine, KCl=potassium chloride, FCS=foetal calf serum

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4.1 MicroRNA expression analysis in RGC-5 cells

To understand whether the lack of GFP::3b-3'UTR downregulation in RGC-5 cells was due to the absence of specific microRNA targeting its 3'UTR, the expression levels of mir-23 and mir-214 were analysed in RGC-5 cells. As reported in section 3.5, these two microRNAs, which target and degrade Brn-3b mRNA, are upregulated following serum removal in the ND7 neuroblastoma cell line. To understand whether a similar modulation occurs in RGC-5 cells, these were treated with staurosporine, KCl or

left untreated and total RNA was extracted and subjected to a northern blot analysis. As shown in figure 4.1, although both the 75 and 22 nucleotide long precursor and mature form of mir-23 are expressed at detectable levels in RGC-5 cells, mir-214 is not detected. Furthermore, in contrast to their upregulation in ND7 cells induced to differentiate via serum removal, neither treatment seems to have any effect on the modulation of mir-23 expression levels in RGC-5 cells.

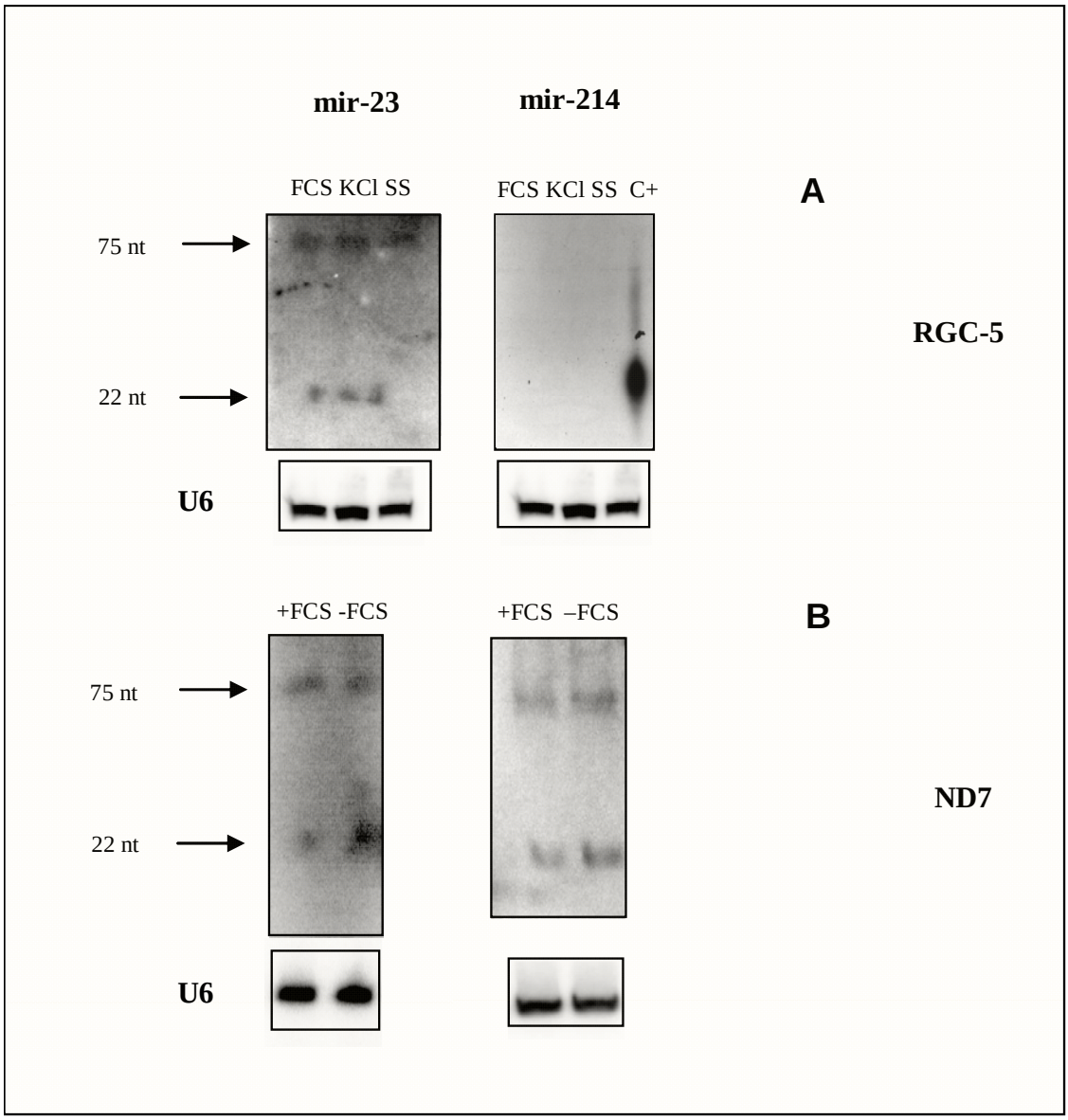

Fig. 4.1: Northern blot analysis of miRNA expression in RGC-5 and ND7 cells. Total RNA was extracted from RGC-5 (A) or ND7 (B) treated as described. RNA was run on polyacrylamide gel and probed with mir-23 or mir-214 probes. Blots were normalized with U6. The 75 nt. and 22 nt. long mature microRNAs are indicated by arrows. The data represent a typical northern blot which were repeated three times.

4.2 Effect of mir-23 and mir-214 on Brn-3b levels in RGC-5 and ND7 cells

To test whether the lack of GFP::3b-3'UTR downregulation was due to the absence of these microRNAs and for the direct role exerted by mir-23 and mir-214 on the stability of the 3'UTR of Brn-3b, RGC-5 cells were transfected with the GFP::3b-3'UTR reporter construct or a GFP empty vector together with synthetic mir-23, mir-214, mir-23 plus mir-214 or a control microRNA. After two days, cells were harvested and subjected to a western blot analysis. As shown in figure 4.2, GFP levels were reduced in RGC-5 cells only upon the co-transfection of the two microRNAs suggesting a co-operative effect.

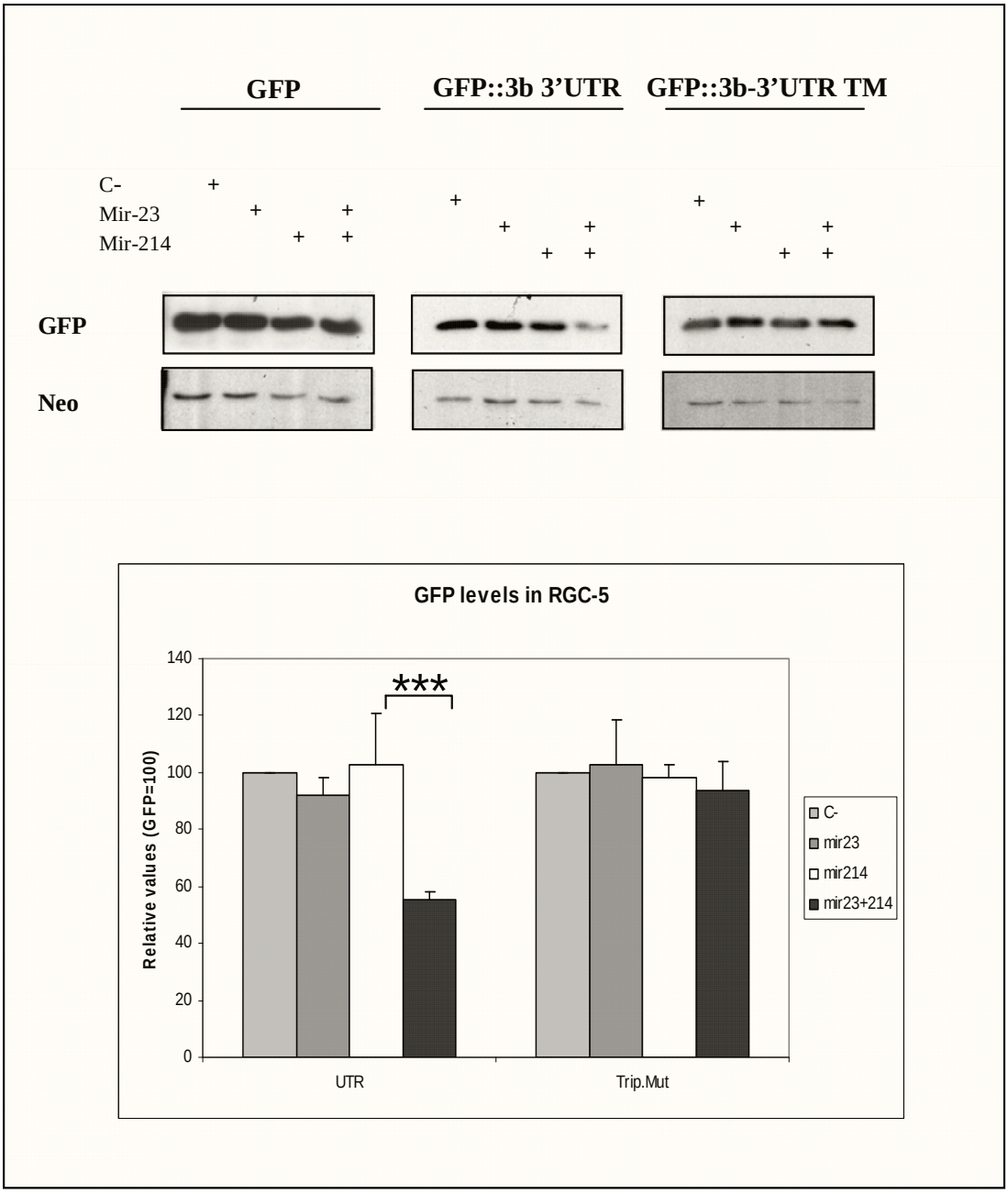

Fig.4.2: Effect of microRNAs on the GFP levels in RGC-5 cells. RGC-5 cells were co-transfected with the indicated constructs and microRNAs. Extracted proteins were subjected to a western blot analysis. The graph represents the average of at least three experiments and the error bars indicate standard error. ***=P<0.001

Conversely, as shown in figure 4.3, the same experiment performed in ND7 cells shows that mir-23 and mir-214 mediate the degradation of the reporter construct harbouring the 3'UTR of Brn-3b both individually and in combination. This, once more, suggests that the chimaeric mRNA is subjected to a differential modulation depending on the cell line in which it is expressed.

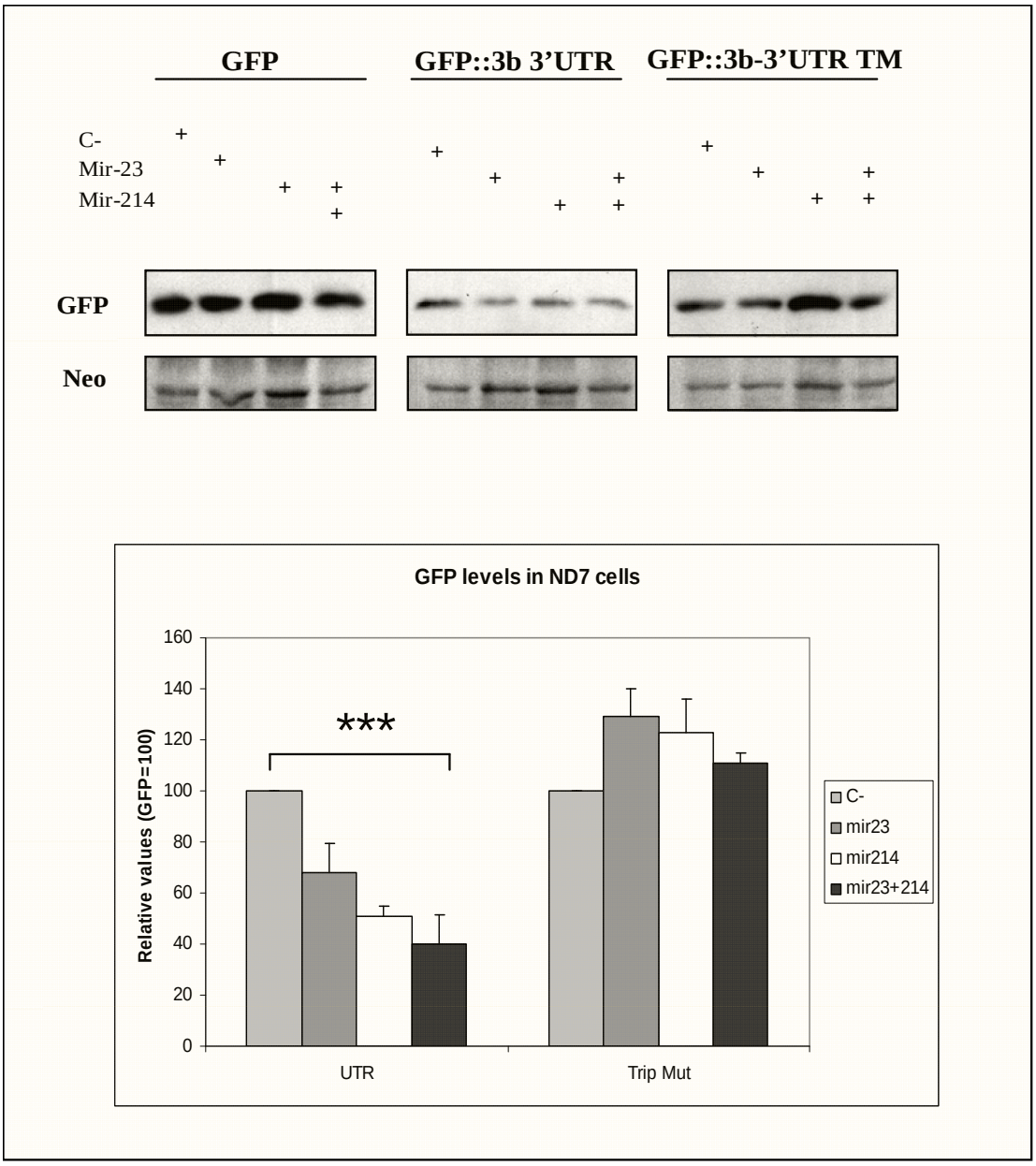

Fig 4.3: Effect of microRNAs on the GFP levels in ND7 cells. RGC-5 cells were co-transfected with the indicated constructs and microRNAs. Proteins were extracted and subjected to a western blot analysis. The graph represents the average of at least three experiments and the error bars indicate standard error. ***=P<0.001

4.3 Effect of mir-23 and mir-214 on endogenous Brn-3a and Brn-3b expression levels.

To confirm the reported findings at a more physiological level, the above experiment was repeated without the presence of the GFP reporter construct and the levels of the endogenous Brn-3b mRNA were measured via realtime RT-PCR. As shown in figure 4.4, the levels of Brn-3b, but not of the closely related Brn-3a transcription factor, are markedly reduced upon the co-transfection of mir-23 and mir-214. This suggests a direct effect of the microRNAs on the 3'UTR of endogenous Brn-3b. Furthermore, endogenous Brn-3b mRNA levels are also reduced upon the transfection of mir-214 but not of mir-23. This is likely due to the endogenous expression of mir-23 in RGC-5 cells which, together with mir-214, triggers Brn-3b degradation. This could also explain why such an effect is not observable on the chimaeric construct, which by being vastly in excess in RGC-5 cells transfected with the peGFP::3b-3'UTR construct, is not degraded by the endogenous levels of mir-23 combined with the exogenously transfected mir-214.

As a control, a northern blot analysis was performed to test whether the result was due to differential microRNAs transfection efficiency but found no differences in the levels of the exogenously transfected mir-23 and mir-214 (data not shown).

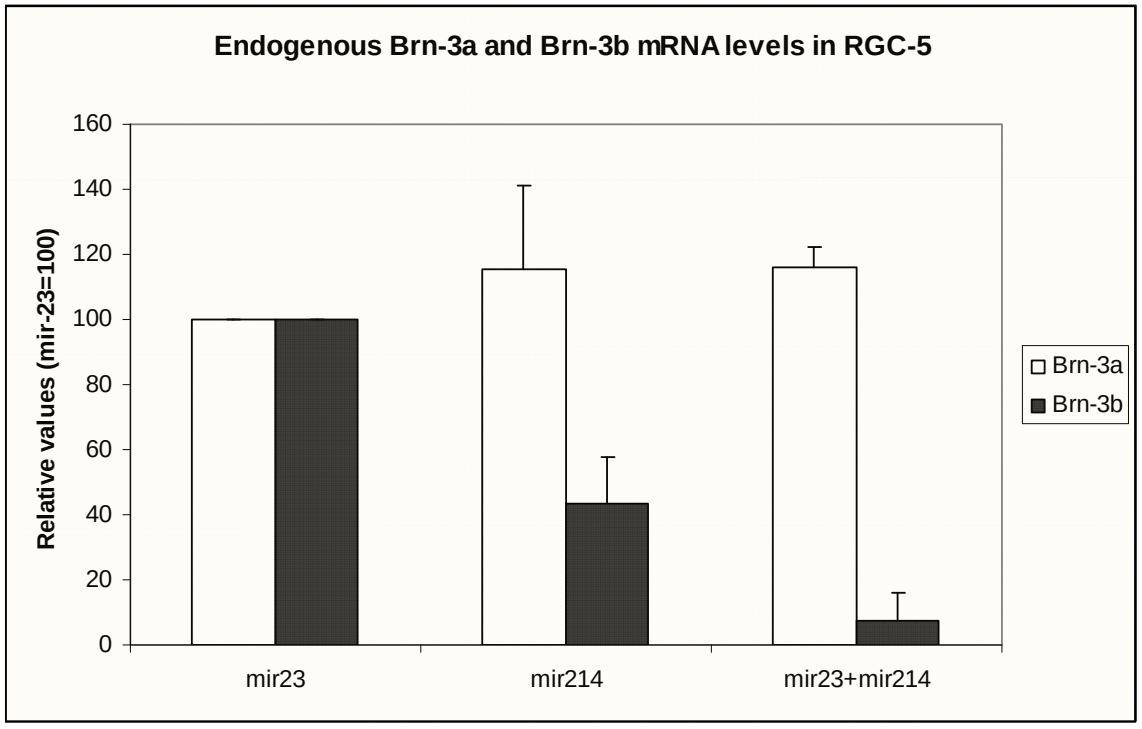

Fig 4.4: Endogenous levels of Brn-3a and Brn-3b mRNA in RGC-5. Total RNA was extracted from RGC-5 cells transfected with the indicated microRNAs, subjected to a cDNA synthesis and used for a realtime RT-PCR reaction to detect the levels of endogenous Brn-3a and Brn-3b mRNAs. The graph represents the average of three experiments.

4.4 Effect of Brn-3b reduction on RGC-5 and ND7 cell survival

As reported before, the deletion of Brn-3b causes RGC death during development (Gan et al. 1996; Erkman et al. 2000; Wang et al. 2000). The data presented in this thesis seems to indicate that the mRNA of Brn-3b is degraded by specific microRNAs in the ND7 but not in the RGC-5 cell line. In order to understand whether this is due to the pro-survival role exerted by Brn-3b in the RGC derived cell line, Brn-3b expression was suppressed and RGC-5 cell viability assessed.

Initially, RGC-5 cells were transfected with mir-23, mir-214, mir-23 plus mir-214 or control miRNA and cell viability was measured by propidium iodide uptake. This compound enters the membrane of dead or dying cells and upon binding DNA its fluorescence increases up to 30 fold compared to the non-bound form. As shown in figure 4.5, mir-214 and mir-23 plus mir-214 induce cell death in RGC-5 cells compared to control.

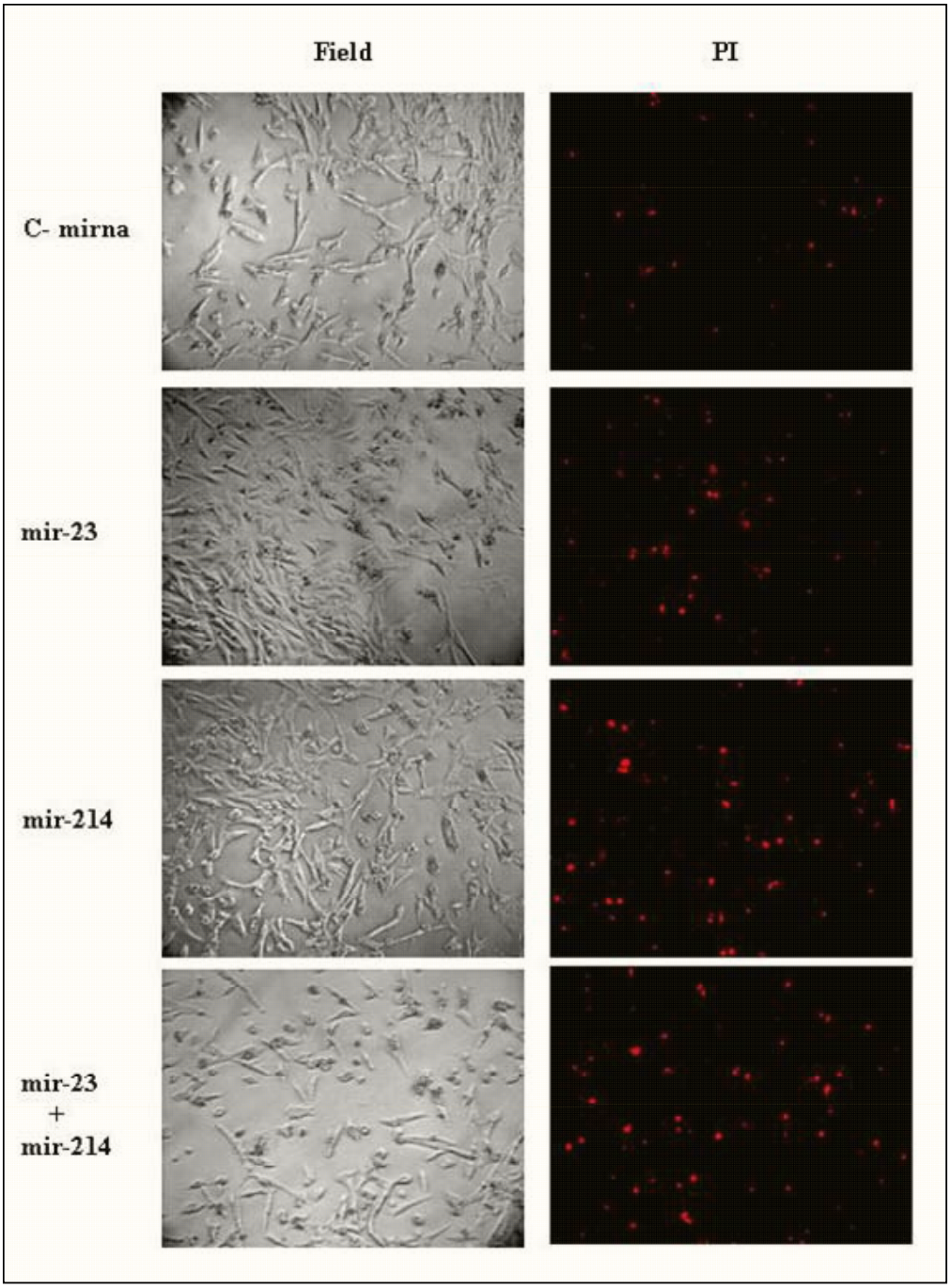

Fig. 4.5: Propidium iodide uptake in RGC-5 cells. RGC-5 cells were transfected with the indicated microRNAs and after two days incubated with propidium iodide. Red nuclei were observed under fluorescent microscope.

In view of the fact that microRNAs can bind several target mRNAs, the cell death assay was repeated with shRNAmir specifically targeting three sites on the coding sequence of Brn-3b. Furthermore, since Brn-3b is not essential for its survival, the ND7 cell line was used as a control. The shRNAmir were transfected in RGC-5 and ND7 cells and the effect on cell survival assessed by propidium iodide uptake measured via flow cytometry analysis. As shown in figure 4.6, increased amounts of shRNA produce an increased amount of cell death in the RGC-5 but not in the ND7 cell line. This suggests that, as well as in primary RGC, Brn-3b plays an essential role in RGC-5 survival but not in the ND7 cell line.

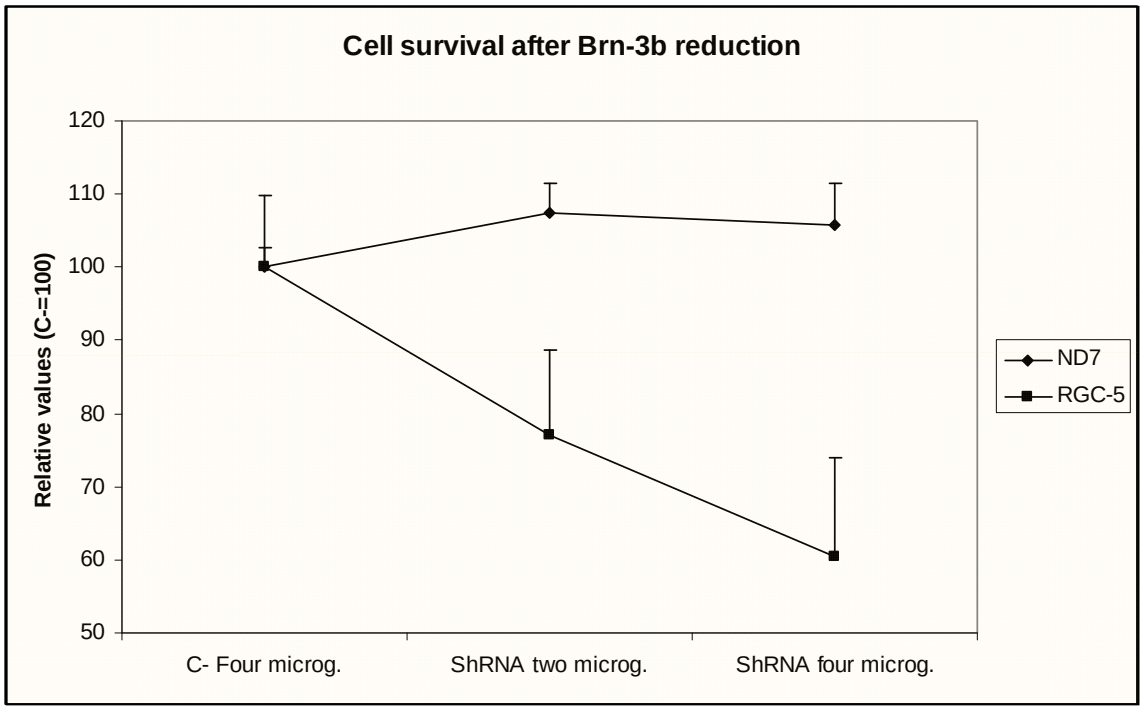

Fig.4.6: RGC-5 and ND7 cell death assay. RGC-5 and ND7 cells were transfected with increased amounts of shRNAmir and their death assessed by propidium iodide uptake as measured via flow cytometry analysis. The data represent cell survival and the graph is the mean of at last four independent experiments. Error bars indicate standard error.

4.5 Discussion

The Brn-3b transcription factor is essential for RGC survival as demonstrated by the loss of up to 70% of RGC in mice lacking the gene (Gan et al. 1999; Erkman et al. 2000; Wang et al. 2000). In chapter 3 of this thesis it has been described how Brn-3b seems to be subjected to down-regulation by two microRNAs (mir-23 and mir-214) in the ND7 and SHSY-5Y neuronal cell line but not in the MCF-7 cell line. In this chapter this analysis was extended and the regulation of Brn-3b by microRNAs was evaluated in an immortalized rat retinal ganglion cell line (RGC-5).

Contrary to previous findings in the ND7 and SHSY-5Y cell line, Brn-3b does not seem to be down-regulated in RGC-5 cells following differentiation. Furthermore, although mir-23 is expressed in RGC-5 cells, differentiating or depolarizing agents such as staurosporine and KCl do not modulate its levels and mir-214 is not even detectable. Due to the presence of a positive control in the northern blot, it is unlikely that the lack of mir-214 expression in RGC-5 is due to a technical issue and therefore the result seems to indicate the existence of a differential regulation of mir-214 in the RGC-5 compared to the ND7 cell line. This differential expression would probably not be of much significance in itself as different cell lines have different gene and microRNA expression profiles but intriguingly, a similar microRNA expression profile, whereby mir-23 is expressed and mir-214 is un-detectable, occurs in primary rat retinas (Arora et al. 2007; Hackler et al. 2010). This suggests that the pattern observed in RGC-5 might reflect a physiological phenomenon. Hackler and colleagues (Hackler et al. 2010) have recently reported that while mir-23 is expressed during mouse retinal development and its expression increases at the adult stage, mir-214 is not expressed at any developmental

stages (Hackler et al. 2010). It has been reported in chapter 3 that Brn-3b is not down-regulated in the MCF-7 cell line. Intriguingly, it has been previously reported that Brn-3b is essential for MCF-7 cell proliferation and invasiveness (Dennis et al. 2001). The reduction of Brn-3b levels via anti-sense leads to a drastic decline of MCF-7 cell growth. Paralleling the microRNA expression profile in the rat retina, mir-23 is expressed in the MCF-7 cell line at low levels while mir-214 is not detectable (Volinia et al. 2006). Furthermore, Kovalchuk and colleagues (Kovalchuk et al. 2008) have shown that while mir-214 is virtually absent, mir-23 is highly expressed in wild type MCF-7 cells (Kovalchuk et al. 2008). Both classes of microRNAs are modulated by the treatment of MCF-7 cells with doxorubicin with a slight increase of mir-214 levels and a slight decrease of mir-23 levels (Kovalchuk et al. 2008).

The regulation of mRNAs by microRNAs is very likely not as straightforward as one microRNA one target mRNA. Several studies (Gaidatzis et al. 2007; Alexiou et al. 2009; Ghildiyal et al. 2009; Orom et al. 2010) offer a much more complicated reality in which the distribution of the number of target sites per miRNA is highly nonuniform. This ranges from an apparent lack of targets for seven miRNAs to 268 targets for *let-7b* (John et al. 2004) , with an average of 7.1 mRNA targets per miRNA (John et al. 2004; John et al. 2006; Ambros et al. 2007). Furthermore it has been also reported (Hon et al. 2007) that the spacing between microRNA target sites and the length of the 3'UTR affects the way the latter is downregulated (Hon et al. 2007; Saetrom et al. 2007). These data suggest that the regulation of mRNAs by microRNAs is of high complexity and that the apparent necessity of both mir-23 and mir-214 to regulate Brn-3b mRNA in RGC-5 cells is very likely just one aspect of Brn-3b regulation.

In the RGC-5 cell line, the modulation of the GFP::3b-3'UTR mRNA and of the endogenous Brn-3b mRNA seems to occur only when both mir-23 and mir-214 are exogenously transfected. In particular, the decreased expression of endogenous Brn3b mRNA upon the transfection of the mir-214 but not of the mir-23 microRNA seems to support the hypothesis that both microRNAs are important for Brn-3b degradation. As a working hypothesis it would be intriguing to speculate that endogenous levels of mir-23 act in concert with the transfected mir-214 to down-regulate Brn-3b. In contrast, the transfection of mir-23 does not induce a similar phenomenon and this is possibly due to the lack of mir-214. Since this phenomenon is not due to a differential transfection efficiency or degradation of the two microRNAs as measured by a northern blot analysis, these data suggest that in this cell line, and perhaps in MCF-7 cells, both mir-23 and mir-214 are required for the downregulation of Brn-3b mRNA. Furthermore, the lack of any downregulation on the GFP::3b-3'UTR construct harbouring the triple mutation which disrupts both the mir-23 and mir-214 binding sites suggests that the reduction of Brn-3b levels is not mediated by, fore instance, the reduction of a protein protecting Brn-3b mRNA but is instead due these microRNAs acting directly on the 3'UTR of Brn-3b.

At the present time it is not known whether the degradation of the 3'UTR of Brn-3b occurs following the binding of both mir-23 and mir-214 or to an indirect effect associated to the over-expression of mir-23 and mir-214 in RGC-5 cells. An mRNA targeted by several microRNAs could undergo, for instance, to a modification of its secondary and tertiary structure (Hofacker 2007; Zhao et al. 2007; Rabani et al. 2008) and this could potentially reduce or enhance its chances of being degraded.

Furthermore, since the transfection of exogenous microRNAs might "flood" the RNAi machinery of RGC-5 cells, it would be intriguing to evaluate the effect of mir-23 and mir-214 on Brn-3b following the modulation of their endogenous levels by acting on their promoters. Conversely, silencing the expression of endogenous mir-23 and mir-214 via specific siRNA could allow the understanding of their specific role on the degradation of Brn-3b mRNA. As mentioned above, although mir-23 and mir-214 are involved in Brn-3b regulation, it is expected that other, not yet identified microRNAs, might be involved in its regulation.

Given the evidence provided in this chapter, it could be speculated that, possibly due to its essential role, the Brn-3b transcription factor could be physiologically protected from degradation by microRNAs in the RGC-5 and MCF-7 cell lines in which plays a pro-survival and pro-growth role, compared to the ND7 and SHSY-5Y cell lines in which, although expressed (Smith et al. 1997; Lee et al. 2005), does not play any essential role.

The next chapter will present preliminary evidence regarding the effect of manipulating Dicer levels in RGC-5 and primary retinal ganglion cells.

Chapter 5

Initial evaluation of the role of Dicer in primary chick RGCs

5.0 Analysis of the 3'UTR of chicken Brn-3b

The results presented in chapter 3 and 4 prompted questions regarding Brn-3b regulation in primary RGCs. Furthermore, given the function of Dicer and generally of PTGR in neuronal development it became interesting to understand what role, if any, it had in RGC development and survival.

In order to study the role played by Brn-3b and its regulation by microRNAs in primary RGC, the chick embryo was chosen as model system because of the abundance of RGCs at different embryonic stages (Prada et al. 1991; Butowt et al. 2000; James et al. 2003)and its easy manipulation.

The Ensembl DNA database (ensemble.org) was used to identify the 3'UTR of chicken Brn-3b (cBrn-3b). This search showed that, despite the evolutionary distance between avians and mammals, this region in the chicken shows a relatively high degree of homology with the murine 3'UTR of Brn-3b (figure 5.0).

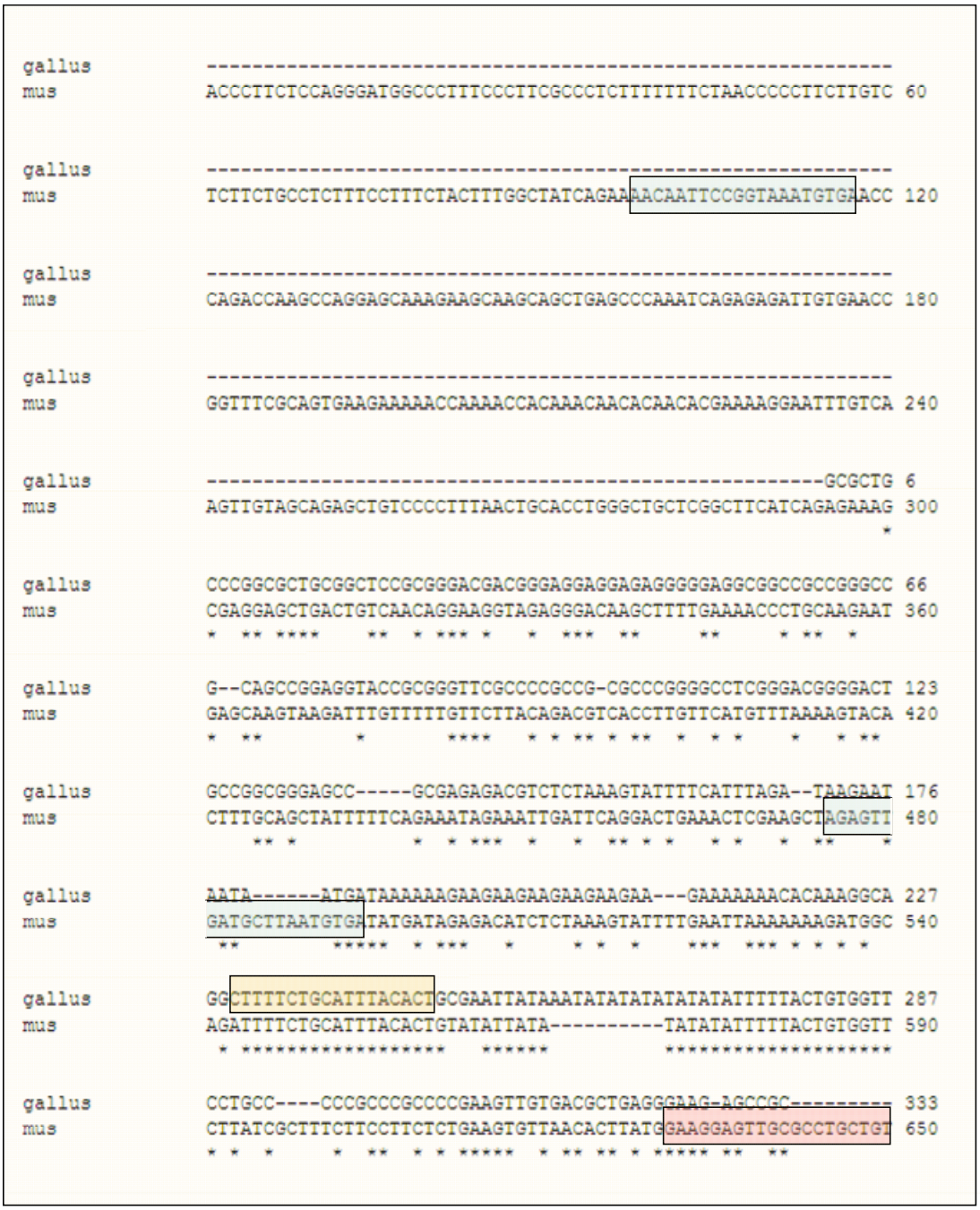

Fig. 5.0: Homology of the 3'UTR of murine and avian Brn-3b. The murine (mus) mir-23 binding sites on the 3'UTR of Brn-3b are highlighted by blue-boxes ("upstream" and "downstream" respectively), the avian mir-23 binding site on the 3'UTR of cBrn-3b (gallus) by a yellow box while the murine mir-214 binding site is labelled with a red box. The asterisks denote nucleotide identity. Continued on the next page.

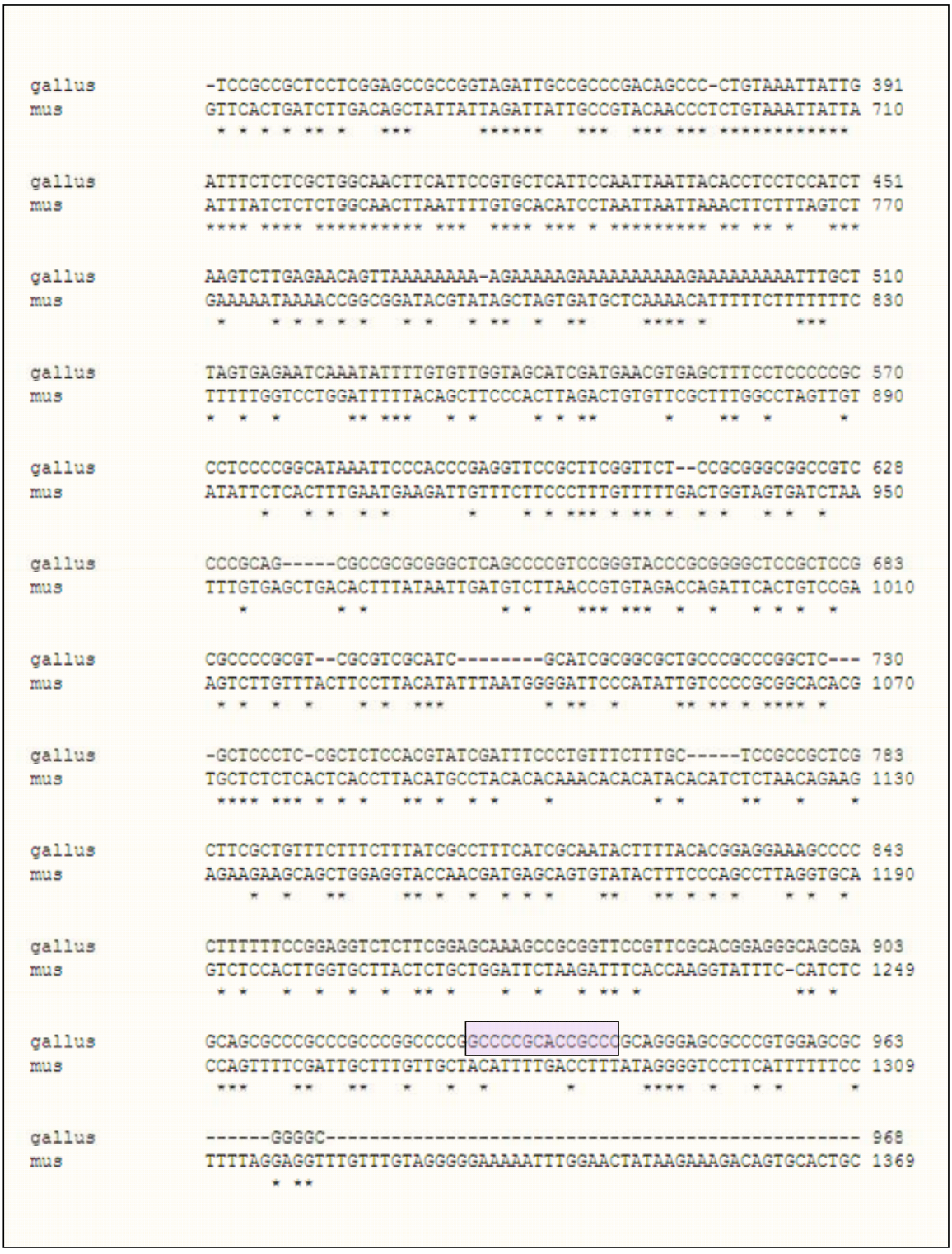

Fig..5.0 (continued): Homology of the 3'UTR of murine and avian Brn-3b. The purple box represents the non–conserved mir-1777 binding site on the 3'UTR of the avian but not of murine Brn-3b.

Although none of the murine-equivalent mir-23 binding sites are present on the 3'UTR of cBrn-3b (figure 5.0 blue boxes), the chick mir-23 has a binding site close to the position of the mouse mir-23 'downstream' site (figure 5.0 yellow box) and this region is highly conserved between the murine and chick sequence (table 5.0).

The mir-214 microRNA is partially conserved on the 3'UTR of cBrn-3b but its most important part, the seven nucleotide 'seed' towards the 3' end of the consensus sequence, is not present on the chick sequence. Intriguingly, a new microRNA, mir-1777, can putatively bind the 3'UTR of cBrn-3b but not the mouse sequence. Although no data are available yet as to its expression, it would be interesting to assess whether it plays any role in the modulation of cBrn-3b.

Mir-23 binding site conservation
on the 3'UTR of chick and mouse Brn-3b

```
3′ GGAAAUCCCUGGCAAUGUGA 5′  Chick mir-23
   * ***  ***   *******
5′ CUUUUC TGCAU-- UUAC A CU 3′  Chick Brn-3b
     *** ** *  *******
5' AACAAUUCCGGUAAAUGUGA 3'  Mouse Brn-3b
     *** ** *  *******
3' CCUUUAGGGACCGUUACACU 5'  Mouse mir-23
```

Table 5.0: Conservation of mir-23 binding site on the 3'UTR of murine and chick Brn-3b.

The *in silico* results concerning the potential regulation of the 3'UTR of cBrn-3b by chick microRNAs has shown i) that the previously described murine mir-23 binding sites are not conserved on the 3'UTR of cBrn-3b, ii) a new potential mir-23 binding site

is present on the 3'UTR of cBrn-3b iii) mir-214 is not conserved on the 3'UTR of cBrn-3b and iv) a different microRNA, mir-1777, putatively targets cBrn-3b.

5.1 Evaluation of microRNA activity in primary RGC

As reported previously, Brn-3b has been found to be expressed in primary chick RGCs (Lindeberg et al. 1997). Due to the uncertain findings concerning its regulation by microRNAs and the lack of any evidence concerning the PTGR of Brn-3b in primary RGCs, the first question to be addressed was to evaluate whether the machinery involved in post-transcriptional gene regulation was operative in this system. For this purpose the chick system was used to assess the regulation of the murine peGFP::3b-3'UTR construct by microRNAs.

Primary chick RGCs were obtained from the trypsinization of E6 retinas. Following their dissociation, cells were plated on PLL/laminin coated coverslips and the following day transfected with the peGFP::3b-3'UTR vector on its own or alongside the mir-23 and mir-214 or a control microRNA to test for potential toxicity. As shown in figure 5.1, the mir-23 and mir-214 microRNA mediated the degradation of the reporter construct in primary chick RGCs as exemplified by the loss of GFP signal in transfected RGCs while the control did not have any effect. This result suggests that the machinery responsible for microRNA-mediated degradation of the reporter construct is expressed and is functioning in primary RGCs.

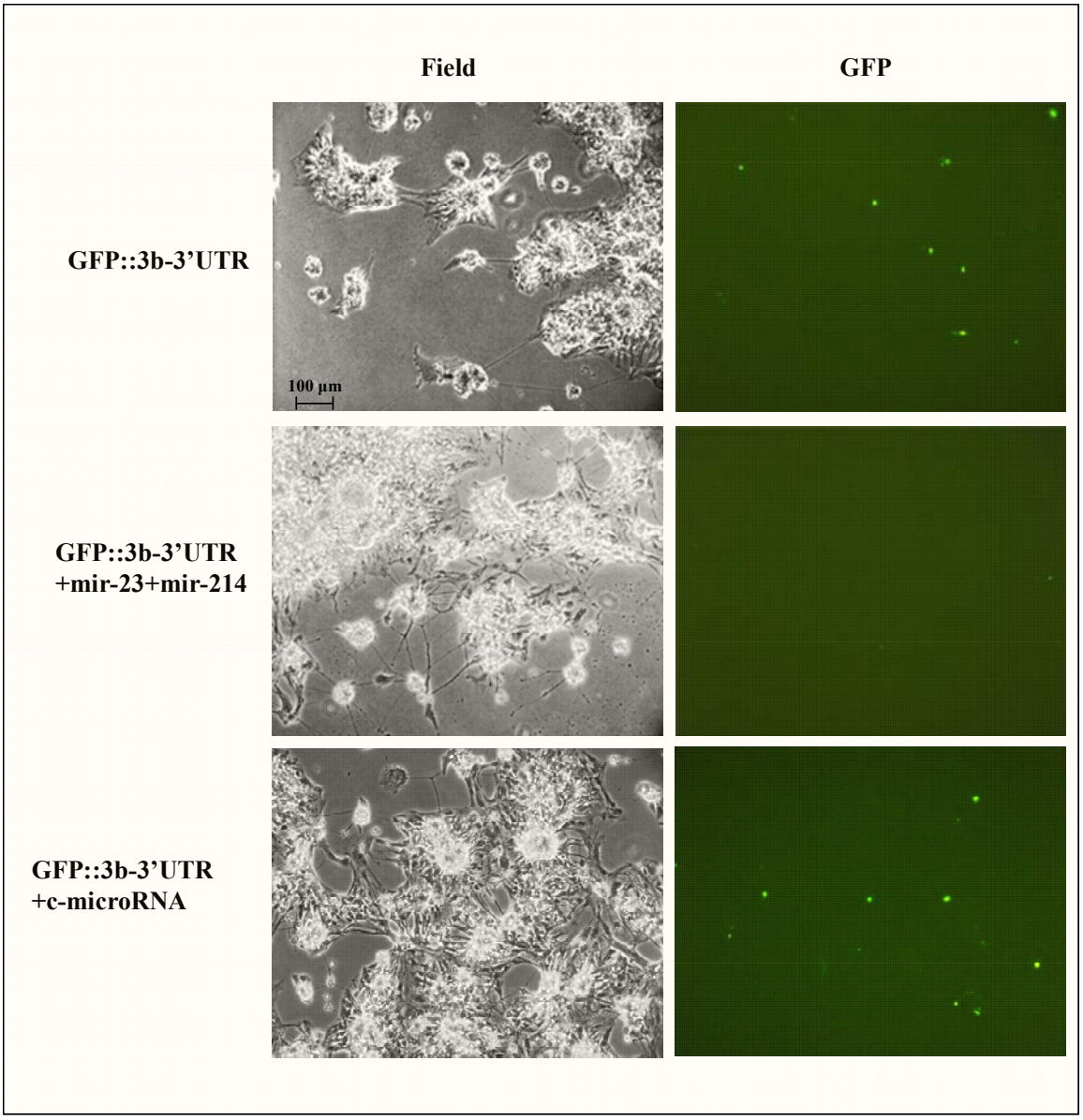

Fig. 5.1: Regulation of the peGFP::3b-3'UTR reporter construct by microRNAs in primary RGC. E6 cells derived from dissociated retina were transfected with the indicated constructs and microRNAs. The levels of GFP have been evaluated by fluorescent microscopy.

Given the data reported above concerning the existence of PTGR in chick retinal cells, the amplification, subcloning and study of the 3'UTR of cBrn-3b would have been the natural evolution from the results presented in this thesis so far. However, in order to

avoid the simple mechanical replication of the experimental approach reported in chapter 3 and 4 of this thesis, it seemed more interesting to start to assess whether PTGR plays a more general role in RGC differentiation and survival and postpone the analysis of cBrn-3b regulation for a later date. For this purpose, due to its key role in PTGR, Dicer levels were experimentally manipulated in RGC-5 and primary RGCs and its sub-cellular localization and effect on specific RGC markers evaluated.

5.2 Expression of Dicer in RGC-5 cells

In section 3.5 it has been reported that the levels of Dicer are modified in ND7 cells induced to differentiate via serum removal. Although it has not been investigated yet, the modulation of Dicer levels might thus affect cellular differentiation. Before evaluating the potential role played by Dicer in the survival and/or differentiation of primary RGCs, its endogenous levels were firstly measured in RGC-5 cells treated with KCl and staurosporine or grown in full serum. As mentioned previously, (section 4.0) staurosporine and KCl are known to respectively affect the differentiation of RGC-5 cells and the intracellular relocation of protein complexes. Contrary to the increased levels of Dicer observed in ND7 cells following differentiation induced by serum removal (figure 3.12, page 98), the differentiation of RGC-5 cells following the treatment with staurosporine induces a reduction of Dicer levels compared to untreated cells (fig 5.2) while KCl has no effect.

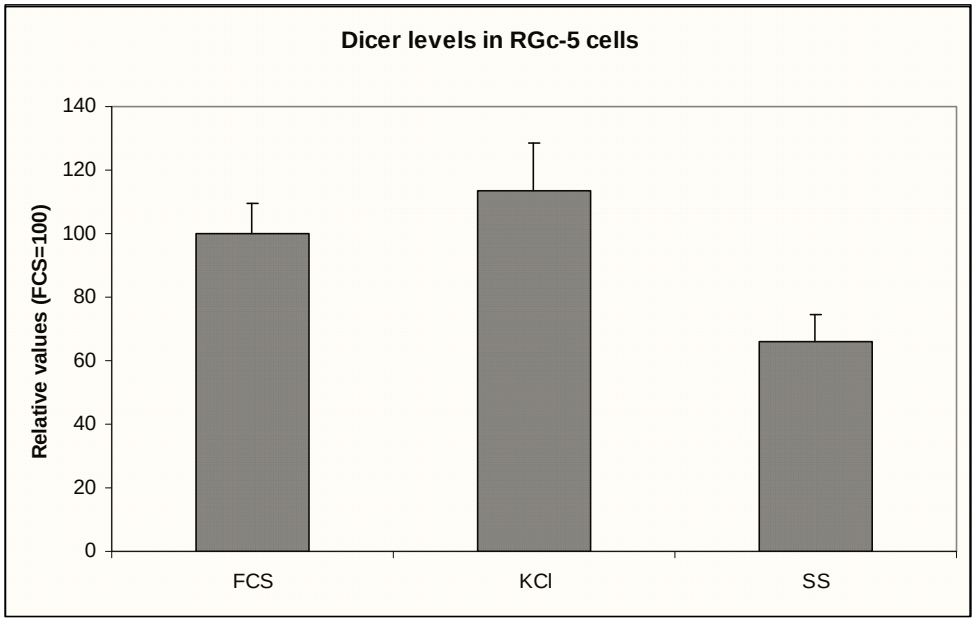

Fig. 5.2: Dicer mRNA levels in RGC-5. Dicer expression was measured via realtime RT-PCR following treatment of RGC-5 cells with the following compounds: KCl=potassium chloride; SS=Staurosporine or FCS=grown in full serum. The experiment was repeated at least three times and the error bars indicate standard error.

In order to confirm and expand the data concerning the regulation of Dicer to the protein level, its intracellular localization was evaluated via an immunofluorescence analysis of RGC-5 cells subjected to the aforementioned treatments. As shown in figure 5.3, Dicer can be identified throughout RGC-5 cell bodies and neurite projections and its expression is slightly reduced in SS-treated cells compared to untreated cells. This experiment confirmed the data obtained via realtime RT-PCR and presented in figure 5.2 whereby SS induces a small down-regulation of Dicer mRNA expression in RGC-5 cells. Furthermore, the treatment with KCl seems to be inducing a relocation of Dicer. As shown in figure 5.3 (middle panel of the left column), its expression is more pronounced in what seems to be the peri-nuclear region compared to untreated or staurosporine-

treated cells (see inset in each panel). At this moment is still not clear whether this relocation has any effect on the function of Dicer.

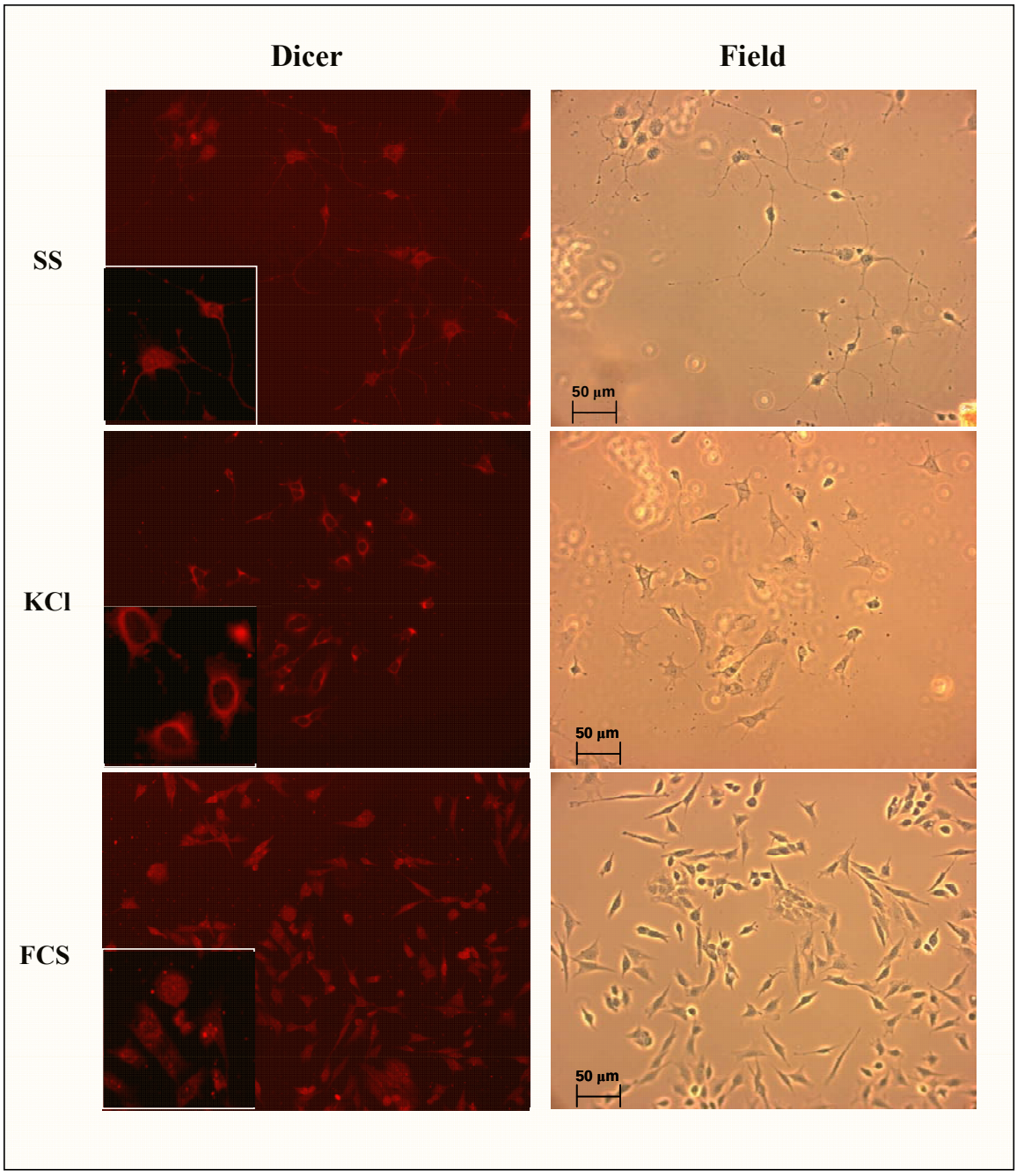

Fig. 5.3: Localization of Dicer in RGC-5 cells. RGC-5 cells were treated with SS, KCl or left untreated and subjected to an immunofluorescence analysis using an anti-Dicer antibody (left column) or visualized via optic microscopy (right column). The magnification in the inset is to show the subcellular localization of Dicer.

5.3 Modulation of Dicer expression levels in RGC-5 cells

To investigate whether Dicer has any role in RGC-5 cell survival and differentiation, its levels were initially increased in this cell line via an expression vector encoding Dicer or conversely reduced via a specific siRNA and its protein and mRNA levels were evaluated via western blot or real-time PCR analysis respectively (figure 5.4). Interestingly, the mRNA levels of Dicer in RGC-5 cells transfected with a Dicer expression vector are more significantly increased than Dicer protein. This might indicate a high turnover of Dicer protein, a low translation rate of its mRNA or a combination of both which might have a direct impact in cell physiology. Conversely, Dicer levels were successfully reduced both at the mRNA and protein level following the transfection with a Dicer specific siRNA.

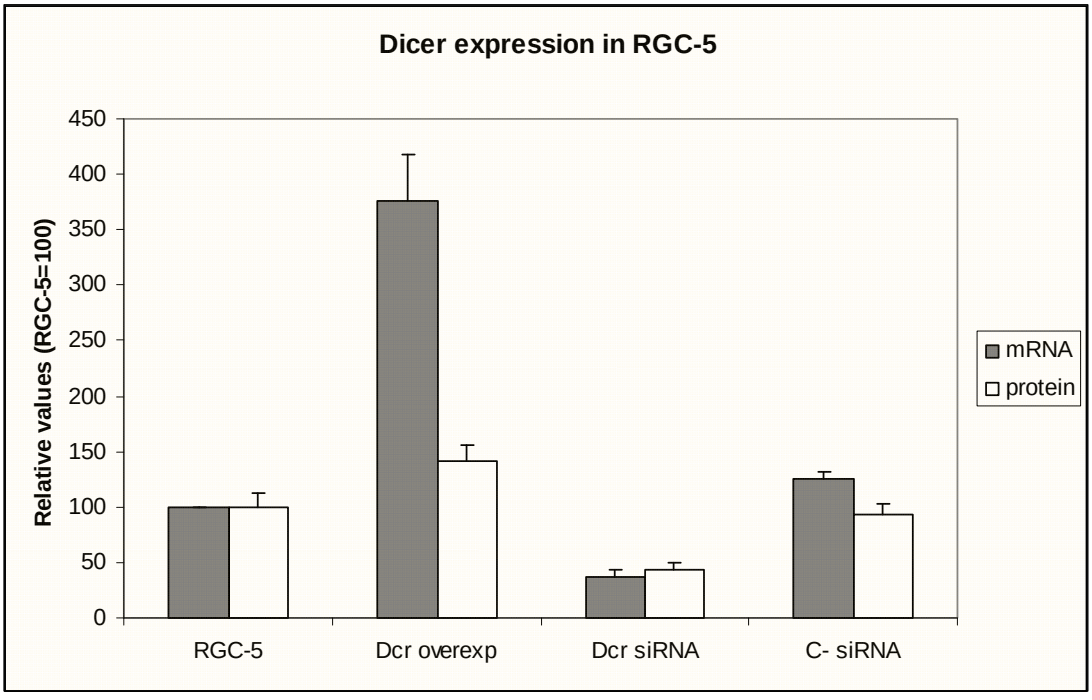

Fig. 5.4: Expression levels of Dicer in RGC-5 cells. RGC-5 cells were transfected with the indicated constructs or siRNA and its expression levels evaluated after two days via realtime RT-PCR. The error bars indicate standard error.

Once more, in order to visually confirm the result, the above experiment was repeated and RGC-5 cells were subjected to an immunofluorescence analysis with an anti-Dicer antibody. As shown in figure 5.5, the expression of Dicer is noticeably increased following its over-expression while the transfection with a Dicer specific siRNA drastically reduces its amount. Conversely a control siRNA does not have any effect on the levels of endogenous Dicer as they appear to be similar to the untreated sample. Although cells lacking Dicer would have been the right control to evaluate the specificity of the anti-Dicer antibody, this is indicated by the differential amount of signal in the Dicer over-expressing cells compared to the ones in which Dicer has been silenced.

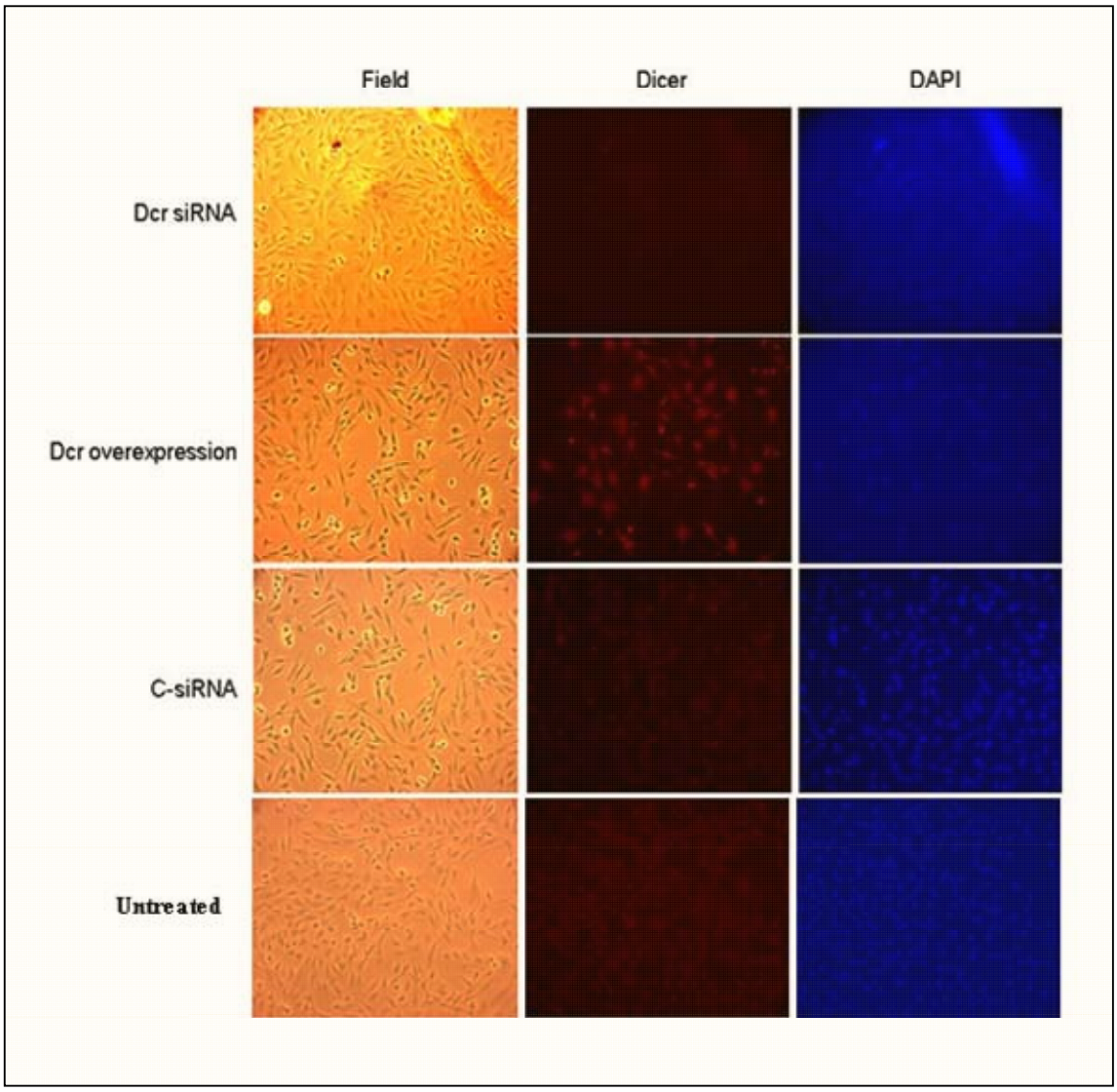

Fig. 5.5: Immunofluorescence analysis of Dicer expression in RGC-5 cells. RGC-5 cells were transfected with a Dicer over-expression vector, a specific siRNA or a control siRNA and subjected to an immunofluorescence analysis with an anti-dicer antibody. Field=light microscopy; Dicer=Dcr antibody; DAPI= staining of nuclei.

5.4 MAP2C expression is affected by the modulation of Dicer's levels

After having successfully modulated Dicer levels in RGC-5 cells, it was next evaluated whether these changes affected the expression of a neuronal differentiation marker such as the microtubule associated protein, MAP2C.

One of the hallmarks of neuronal differentiation is the extension of a network of neurites which require the formation and remodelling of the cytoskeleton for their elongation. MAP2C plays an essential role by stabilizing microtubules involved in the formation of the cytoskeleton (Lieven et al. 2007). This protein is a marker of primary RGCs and RGC-5 cell differentiation (Lieven et al. 2007) and is expressed during neuronal development (Garner et al. 1988; Lieven et al. 2007).

RGC-5 cells were thus transfected with a Dicer expression construct or specific siRNA as described previously and the levels of MAP2C analysed via realtime RT-PCR. Intriguingly, the modulation of Dicer levels has an effect on the expression of MAP2C in RGC-5 cells whereby an increase in Dicer levels produces a corresponding increase in the expression of MAP2C. Similarly, decreased Dicer levels produce a reduction in MAP2C levels. No differences were found following the transfection of a control siRNA suggesting specificity of the effect.

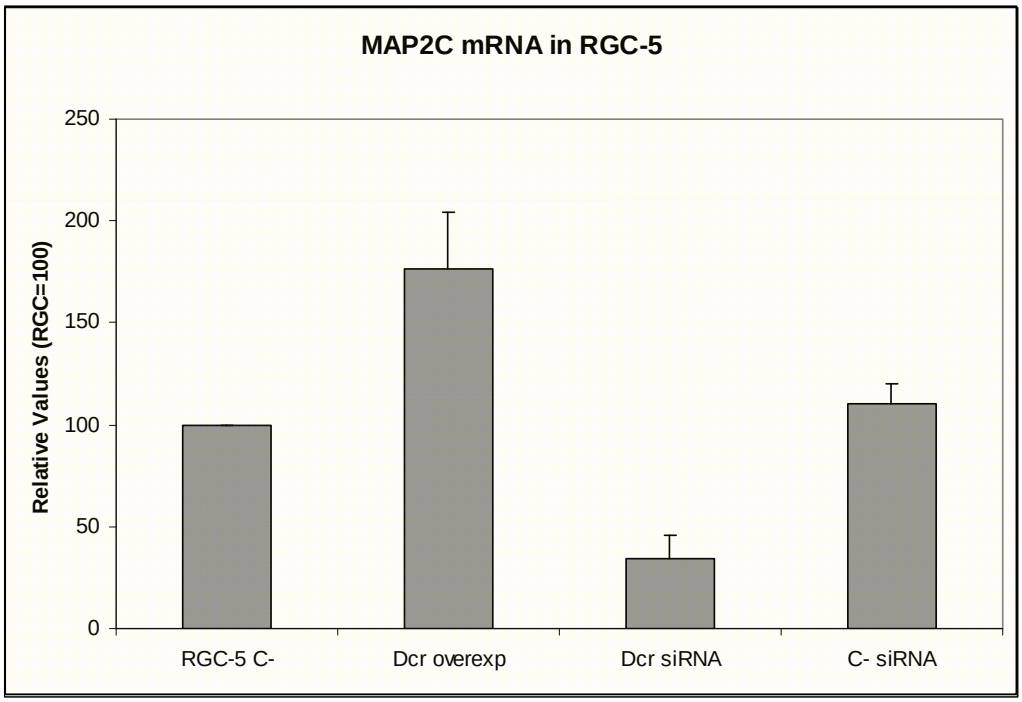

Fig. 5.6: MAP2C levels in RGC-5 cells. Cells were transfected with the indicated constructs and siRNA and after few days total RNA was subjected to realtime RT-PCR with primers specific for MAP2C and its values normalized with GAPDH. The error bars indicate standard error.

5.5 Cultivation of primary embryonic retinal strips

Following the identification of Dicer in RGC-5 cells and its effect on the expression of the RGC differentiation marker MAP2C, the next step was to evaluate whether Dicer is similarly expressed and the manipulation of its levels in primary RGCs has a similar effect to the one observed in RGC-5 cells.

Primary E7 chick retinal strips were prepared as described in section 2.7 of this thesis and grown on PLL/laminin coated-coverslips as exemplified in figure 5.7.

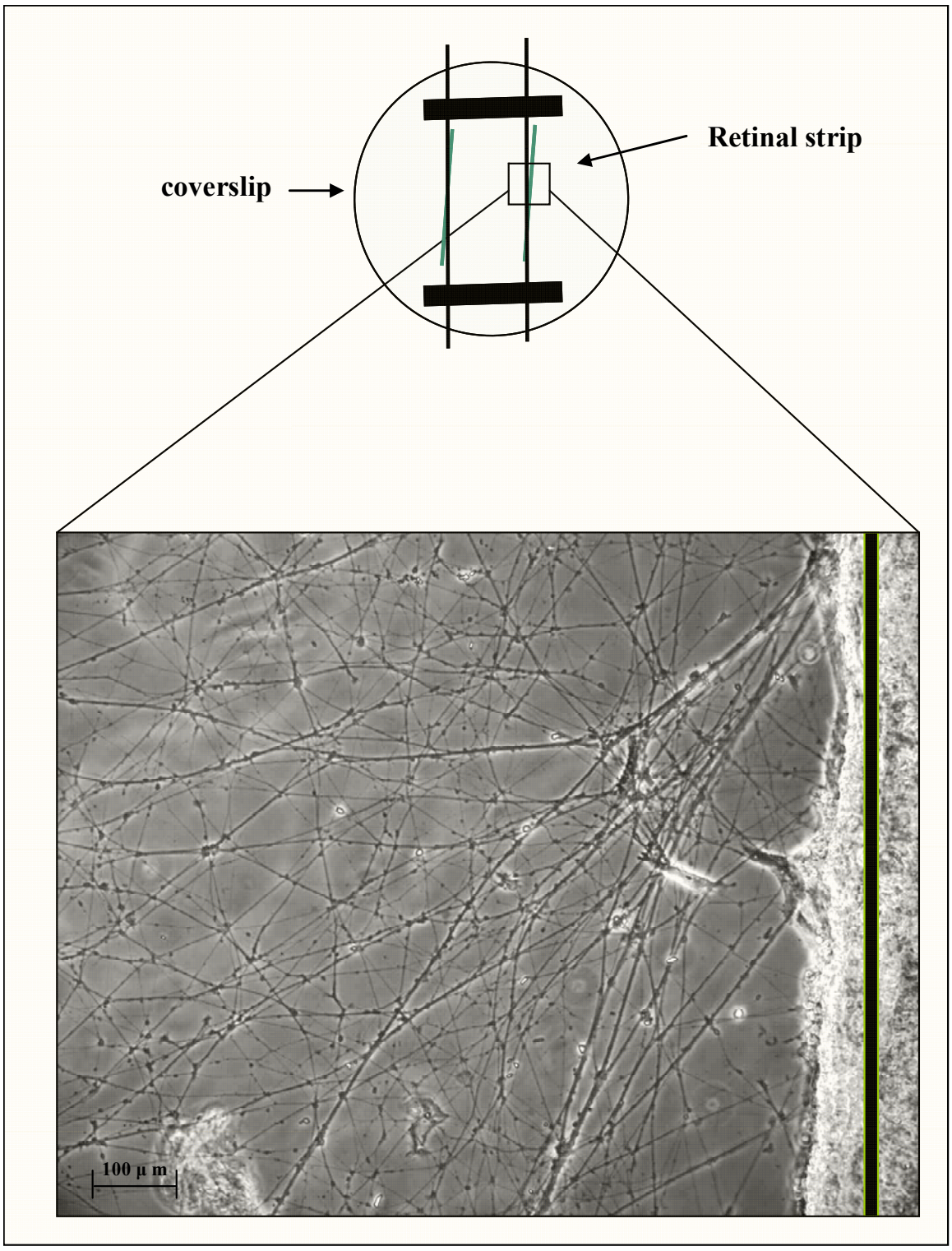

Fig. 5.7: Neurite outgrowth from primary E7 RGCs. Microphotograph of a typical neurite outgrowth from RGCs of an explanted E7retinal strip on PLL/Laminin coated coverslips. Refer to section 2.6 of the material and methods for a detailed description of the technique.

Embryonic stage 7 (E7) was chosen for several reasons. Firstly, peak production of chick RGCs occurs around E7, secondly their axons are fully developed and by the end of E7 reach and innervate the tectum (superior colliculum in mammals) and lastly E7 chick embryos are relatively easy to manipulate.

Primary retinal strips were firstly subjected to immunofluorescence analysis using an anti-actin antibody (fig 5.8). This showed that the neurite arborisation structure of primary retinal strips had been preserved during their manipulation and that actin could be detected as expected throughout cell bodies and their neurites.

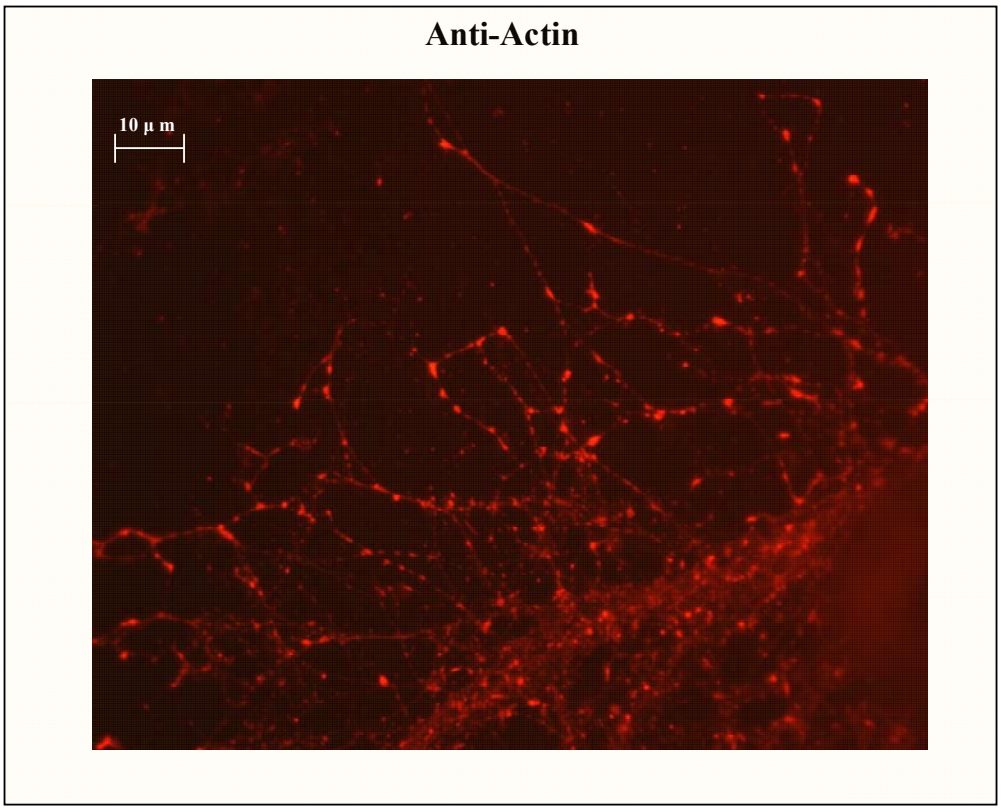

Fig. 5.8: Immunofluorescence analysis of E7 RGCs with anti-actin antibody. Retinal strips were fixed and permeabilized as described in the material and method section, probed with anti-actin antibody and photographed under fluorescent light.

5.6 Expression of Dicer at different embryonic stages

Next, the expression of Dicer was evaluated at different embryonic stages in the chick retinal strips. As shown in figure 5.9, Dicer is expressed at all tested embryonic stages. As a control, the bottom panel shows an immunofluorescence assay performed without primary antibody.

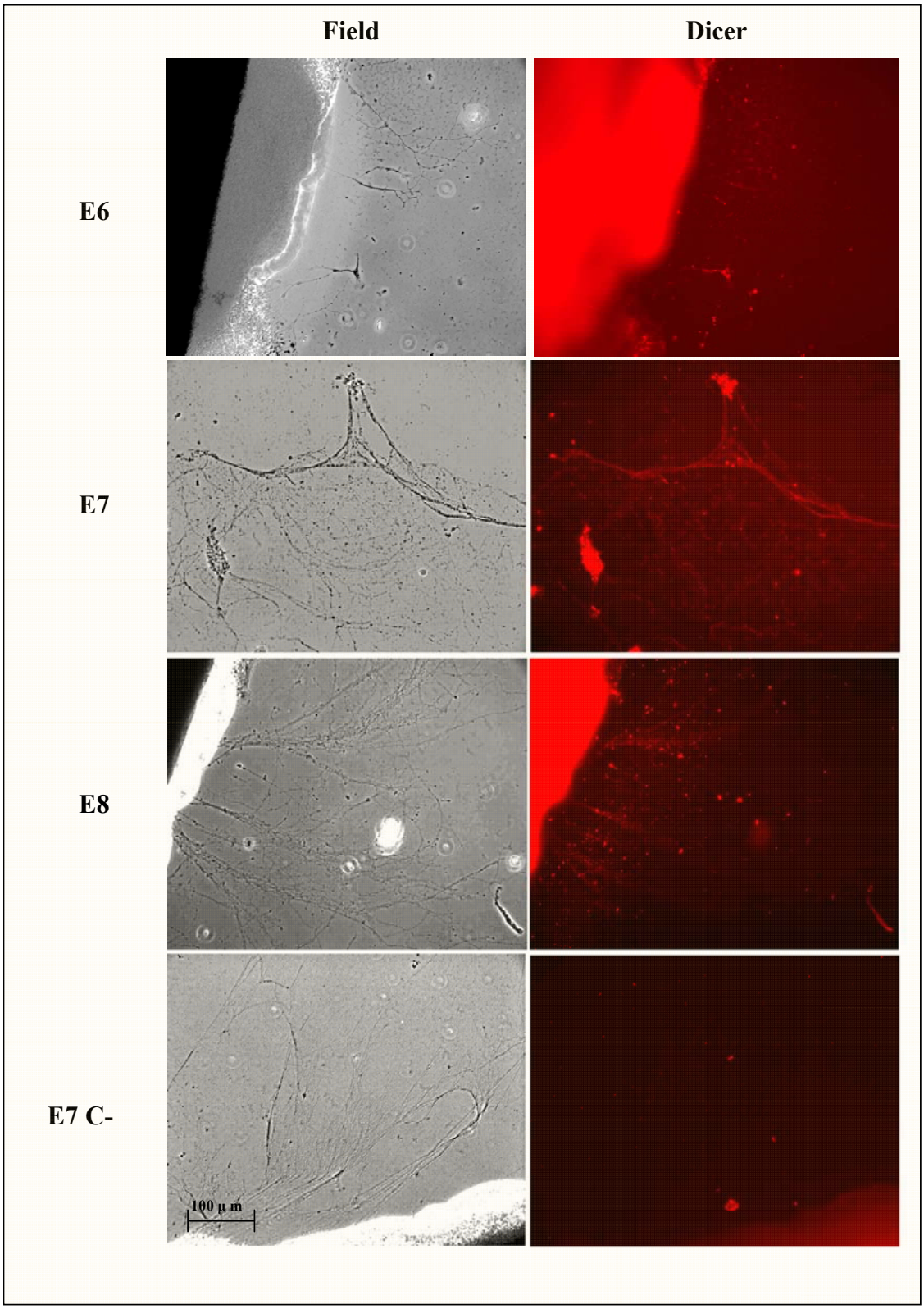

Fig. 5.9: Expression of Dicer at different chick embryonic stages. Retinal strips from embryonic stages E7 and E8 have been treated as described previously. The C- control consists of an immunofluorescence on E7 retinal strip performed without the primary antibody.

Intriguingly, as reported previously in mice (Hengst et al. 2006), Dicer can be found both in cell bodies and throughout almost the entire length of neurites. This suggests that the activity of Dicer in the production of mature microRNAs might be required close to the synaptic terminal where mRNAs and ribosomes have already been identified(Steward et al. 1982) . Furthermore, and as also measured via realtime RT-PCR later in paragraph 5.7 (Fig. 5.10), the expression levels of Dicer seem to remain constant from E6 to E8. At this moment it is still not known whether its expression levels vary throughout chick RGC development.

5.7 RGCs differentiation markers during development.

Before assessing the role Dicer might play in neurite extension and general RGC differentiation, the mRNA expression levels of the differentiation markers of primary RGCs were evaluated at different embryological stages. E6, E7 and E8 retinas were grown on coverslips as described previously and the levels of MAP2C, cATH5 and Brn-3b measured via realtime RT-PCR. While the role of Brn-3b in RGC development has been reported previously in this thesis, cATH5 is a basic helix-loop-helix transcription factor essential for RGC development (Ma et al. 2004). As shown in figure 5.10 the levels of MAP2C increase during embryonic development. This is expected considering the role of MAP2C in the establishment of the neurite network.

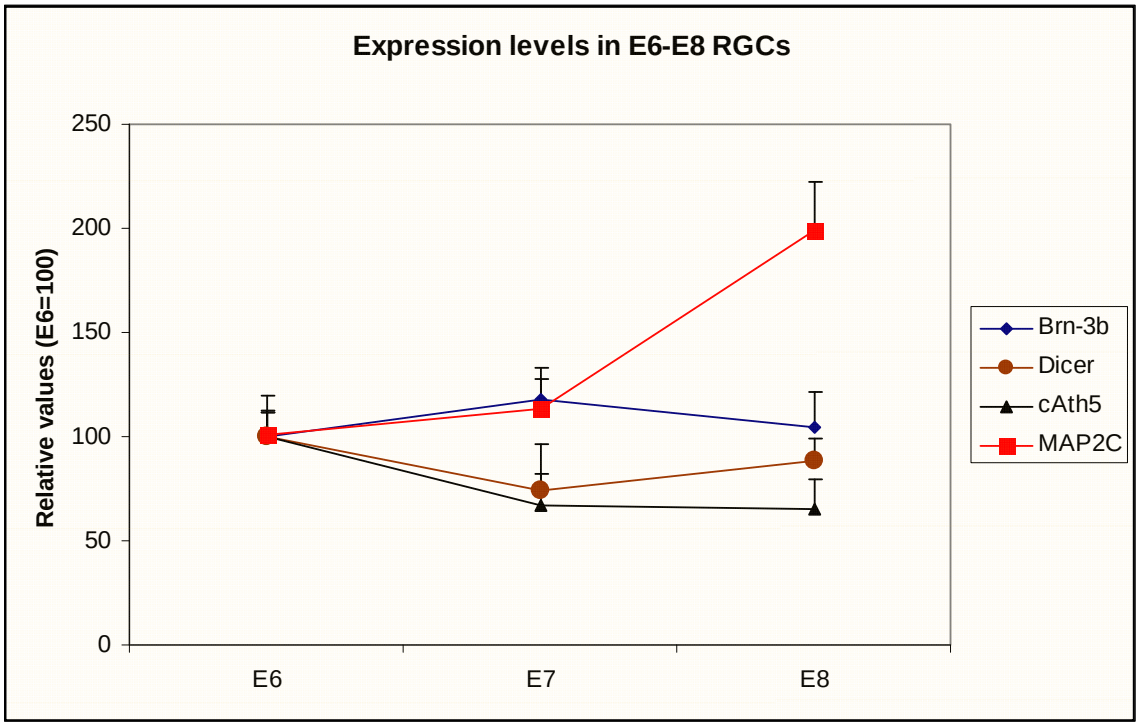

Fig. 5.10: Expression levels in primary RGCs. E6, E7 and E8 primary retinas were dissociated and grown on coverslips. The levels of Brn-3b, Dicer, cATH5 and MAP2C were evaluated by realtime RT-PCR and normalized with GAPDH. Error bars indicate standard error.

Intriguingly, the levels of Brn-3b, cATH5 and Dicer mRNA were not increased during this time-span. This confirms previously published data which describes the beginning of decline of cATH5 expression by E7.5 (Liu et al. 2001) and of Brn-3b by E15.5 in the mouse (E8.5 in the chick) (Pan et al. 2005). The expression levels of Dicer mRNA seem virtually unchanged in the observed time-frame.

5.8 Effect of manipulation of Dicer levels on RGCs differentiation markers

Next, in order to evaluate whether Dicer played any role in modulating the expression levels of MAP2C as demonstrated in the RGC-5 cell line, its expression was artificially elevated in primary RGCs via an over-expression vector. After having confirmed that Dicer mRNA levels were actually increased in this experiment, the mRNA levels of Brn-3b, MAP2C and cATH5 were also measured via realtime RT-PCR. As shown in figure 5.11, the levels of MAP2C are increased following the over-expression of Dicer paralleling the findings in the RGC-5 cell line. Intriguingly the levels of Brn-3b are also increased while the expression of cATH5 is reduced in Dicer over-expressing cells.

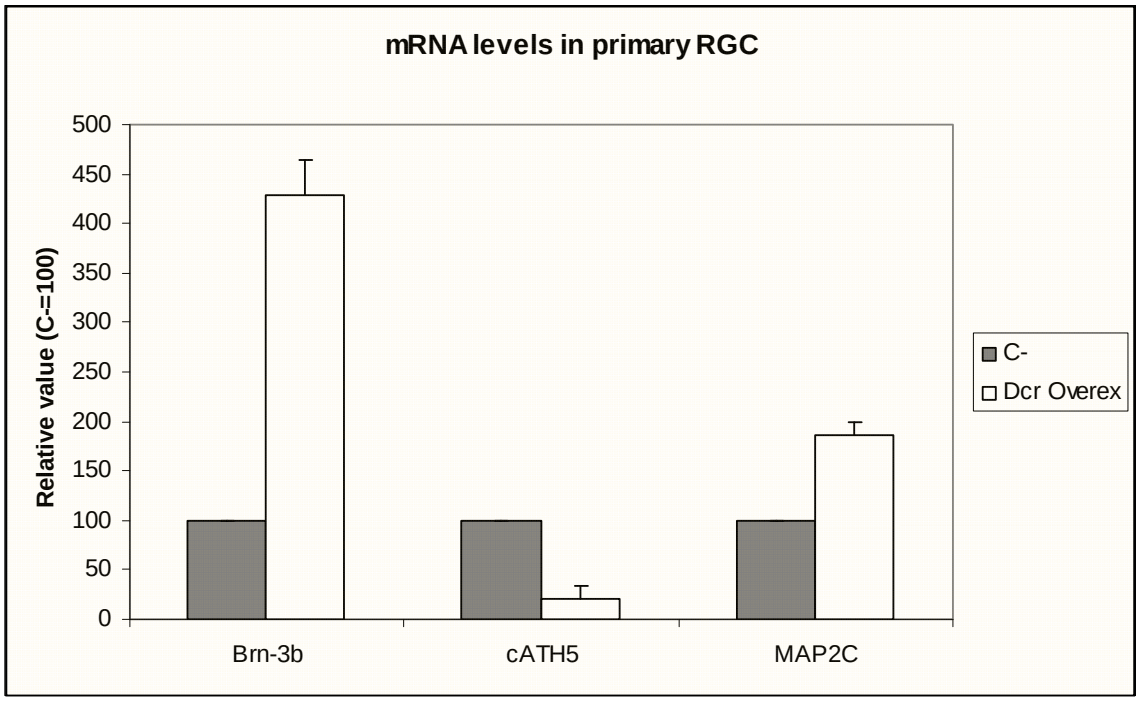

Fig. 5.11: MAP2C levels in primary RGCs. Dissociated E7 retinas were transfected with a Dicer overexpression vector and MAP2C RNA levels evaluated via realtime RT-PCR and normalized with GAPDH. Error bars indicate standard error.

This result shows that Dicer, via an unknown mechanism, seems to be involved in RGC differentiation as measured by the variation in the expression levels of RGC-differentiation markers following its increased expression.

5.9 Discussion

Subsequent to the analysis of the regulation of the 3'UTR of murine Brn-3b in several cell lines, a similar evaluation was attempted in regard of the 3'UTR of the chick Brn-3b. The analysis of this sequence showed that, despite a relatively high homology between the murine and chick 3'UTRs, the microRNA binding sites are poorly conserved. In particular the first 300 nucleotides of murine 3'UTR of Brn-3b, where the mir-23 'upstream' binding site is localized, are not present in the chick. Intriguingly, although the chick mir-23 is different from its murine counterpart, the 3'UTR of the cBrn-3b has a mir-23 binding site which is situated not far from the murine mir-23 'downstream' site. Whether this site has any physiological relevance in the regulation of Brn-3b in RGCs, remains to be established. With regard to the other microRNAs involved in the regulation of the 3'UTR of Brn-3b, mir-214, it is only moderately conserved and the region encompassing the "seed" is not present in the chick 3'UTR. In contrast, a different putative microRNA binding site (mir-1777) was identified. Future work will establish whether this site has any physiological function.

Overall the analysis of microRNAs in the chick genome is still in its early stages compared to their identification and analysis in mammals (Sevignani et al. 2006; Glazov et al. 2008). This might also explain why the 3'UTR of the chick Brn-3b seems to be targeted by fewer microRNAs than the 3'UTR of murine Brn-3b. Alternatively it could be possible that the regulation by microRNAs increased in complexity during evolution (Xie et al. 2004; Grun et al. 2005; Chapman et al. 2007) and hence more microRNAs are produced in mammals compared to avian species (Lee et al. 2007).

In order to test whether primary RGCs contain the machinery involved in PTGR, these cells were transfected with the construct harboring the 3'UTR of murine Brn-3b along with various microRNAs. The 3'UTR of murine Brn-3b was degraded by murine mir-23 and mir-214 suggesting that PTGR is active and functioning in primary chick RGCs.

Due to the presence of Dicer in synaptic terminals (Jin et al. 2004; Siomi et al. 2004; Giraldez et al. 2005; Hengst et al. 2006) it was also assessed whether the manipulation of its levels had any role in the differentiation of RGCs. Firstly a time-course analysis of Dicer expression was performed in E6, E7 and E8 primary RGCs. This showed that, while the levels of Dicer seem to remain constant in this time-frame, the expression of MAP2C increases while the levels of cATH5 and Brn-3b slightly decrease. This result is in agreement with previous findings which show a reduction of Brn-3b an of cATH5 levels during RGC development (Liu et al. 2001; Pan et al. 2005).

In order to further evaluate whether Dicer plays any role in RGC differentiation, its levels were artificially manipulated in RGC-5 cells and in primary E7 RGCs. The over-expression of Dicer in both the RGC-5 cell line and in primary RGCs led to increased levels of MAP2C. Conversely, the reduction of Dicer levels via siRNA led to reduced amount of MAP2C in RGC-5 cells. The elevated levels of MAP2C, a protein involved in the stabilization of the cytoskeleton and neurite outgrowth (Lieven et al. 2007), suggest that Dicer might actually have a role in neuronal differentiation as also suggested previously (De Pietri Tonelli et al. 2008; Kawase-Koga et al. 2009). This is also suggested by its effect on two transcription factors, Brn-3b and cATH5, both involved in the development of RGCs whereby the over-expression of Dicer in E7 RGCs

is associated with increased expression of Brn-3b and with decreased cATH5 levels. These results indicate that, directly or indirectly, Dicer may be involved in RGC differentiation. A possible explanation of this result is that the modification of Dicer levels affects the production of microRNAs modulating the expression of specific mRNAs involved in differentiation. Whilst mir-23 and mir-214 are involved in the degradation of Brn-3b, the over-expression of Dicer might, for instance, increase the production of microRNAs which target inhibitors of Brn-3b transcription and of other mRNAs involved in RGC differentiation. An opposite scenario, whereas microRNA target cATH5 activators, might instead be at the basis of cATH5 down-regulation in Dicer over-expressing cells. Besides the modulation of MAP2C, Brn-3b and cATH5, is likely that the modulation of Dicer levels indirectly affects the expression of several other mRNAs involved in RGC development. Although at the present time it is not clear how the manipulation of Dicer levels might affect the expression of the analyzed mRNAs, it would be very interesting to perform a microRNA and mRNA profiling of differentiating RGCs and of RGCs over-expressing Dicer.

Chapter 6

Conclusion and future work

The regulation of transcription via the modification of DNA packaging (Gatti et al. 1992; Dernburg et al. 1996; Richards et al. 2002; Coulthard et al. 2003; Richards 2008) and via the intervention of specific proteins such as transcription factors (Lee et al. 2000; Latchman 2002) are all mechanisms aimed at insuring that the genetic material is transcribed into RNA only when needed for specific cellular functions. Messenger RNAs are also subjected to a control of their turnover and/or translation via several mechanisms (Kruys et al. 1988; Andrei et al. 2005; Barreau et al. 2005). Furthermore proteins can be subjected to post-translational modification which alters their conformation and activity (Lamph et al. 1990; Chou et al. 1995; Han et al. 1997; Niklinski et al. 2000) and/or differential degradation which modifies their intracellular abundance according to cellular needs (Marquardt et al. 1992; Roth et al. 2008).

The role played by post transcriptional gene regulation in the modulation of the expression of the Brn-3b transcription factor in several neuronal cell lines, was explored in this thesis as an example of another mechanism involved in controlling gene expression. Brn-3b is expressed and plays an essential role in the survival of retinal ganglion cells (Gan et al. 1996; Erkman et al. 2000; Wang et al. 2000; DeCarvalho et al. 2004; Qiu et al. 2008; Badea et al. 2009).

The analysis of the 3'UTR of this transcription factor had evidenced its high degree of conservation amongst different species thus suggesting it might play a still unidentified role in the regulation of its mRNA. It was thus interesting to assess whether, beside its transcriptional regulation, Brn-3b could be subjected to post-transcriptional gene regulation. Initial experiments showed that *in cis* regulatory sequences are likely to be playing a role in the modulation of a reporter construct harboring it in the ND7

neuroblastoma cell line. In particular the differentiation of this cell line via serum removal induces an even stronger degradation of the reporter construct containing the 3'UTR of Brn-3b. This result parallels the modulation of endogenous Brn-3b in ND7 cells whereby the differentiation of this cell line triggers an increase of the closely related Brn-3a transcription factor and a reduction of Brn-3b (Budhram-Mahadeo et al. 1995; Smith et al. 1997). Intriguingly a reduction of the levels of a reporter construct harboring the 3'UTR of Brn-3b was observed in the other neuronal SHSY-5Y tested cell line but not in the breast-cancer derived MCF-7 cell line. This result was interpreted as a working hypothesis that the regulation of the 3'UTR of Brn-3b could be a neuron-only phenomenon. Subsequent analysis proved this hypothesis to be incorrect.

The analysis of the 3'UTR of Brn-3b showed that it contained several putative microRNA binding sites. These were individually mutagenised to assess for their specific role in the degradation of the reporter construct. Two microRNAs, mir-23 and mir-214 were shown to be putatively involved in the degradation of Brn-3b.

MicroRNAs are short RNA molecules that, by binding to target sequences generally situated in the 3'UTR of mRNAs (Lai 2002; Bartel 2004; Rajewsky et al. 2004; Yang et al. 2005), induce their degradation or inhibition of translation (Belostotsky 2004; Cullen 2004; Filipowicz et al. 2005; Jabri 2005; Liu et al. 2005; Rehwinkel et al. 2005). MicroRNAs thus play an essential role in post-transcriptional gene regulation. They are synthesized from within the genome of virtually every organism (Finnegan et al. 2003; Allen et al. 2004; Altuvia et al. 2005; Onishi et al. 2005; Smalheiser et al. 2005; Ying et al. 2005; Zhang 2005) and after a process of maturation which occurs in the nucleus and in the cytoplasm of cells, they acquire their mature and active form (Lee et al. 2002; Cai

et al. 2004; Denli et al. 2004; Murchison et al. 2004; Saito et al. 2005). Following their quasi-serendipitous discovery, the number of identified microRNAs and of their targets has been constantly increasing (Smith 2005; Wang et al. 2005; Corcoran et al. 2009). This, alongside their common evolutionary conservation (Ohler et al. 2004; Altuvia et al. 2005; Zhang et al. 2006), has set microRNAs as potential key players in several aspects of cellular physiology and pathology.

The identification of two microRNAs directly involved in the regulation of the Brn-3b transcription factor triggered further investigation by analyzing their role in retinal ganglion cells where Brn-3b plays an essential role. This study, presented in chapter 4 of this thesis, brought interesting results and speculations. The reporter construct containing the 3'UTR of Brn-3b did not seem to be regulated in the RGC-5 immortalized retinal ganglion cell line as in the other neuronal cell lines. This led to the analysis of the expression levels of the mir-23 and mir-214 in RGC-5 cells. It had been previously shown that both microRNAs are expressed in ND7 cells and that their expression is modulated following differentiation stimuli applied to the cells. Contrary to previous findings in ND7 cells, mir-214 was not detectable in the RGC-5 cell line and mir-23, although expressed at detectable levels, was not upregulated following RGC-5-specific differentiation stimuli. Furthermore, the degradation of the GFP::3b-3'UTR reporter construct mRNA and of endogenous Brn-3b mRNA, seemed to occur only when mir-23 and mir-214 are simultaneously transfected in RGC-5 cells.

This result disproved the working hypothesis that the regulation of Brn-3b was a neuron-only phenomenon and prompted instead a new hypothesis. Could it be possible that, due to the pro-survival role played by Brn-3b in retinal ganglion cells its mRNA is

protected from microRNA-mediated degradation in the immortalized RGC-5 cell line? If that is the case, what is the microRNA expression profile in the primary RGCs? Is Brn-3b mRNA protected from microRNA-mediated degradation during development? The experiments presented in this thesis suggest that Brn-3b might be differentially subjected to microRNA-mediated degradation in different cell lines. This seems to be accomplished by the lack of expression of one of the two microRNAs (mir-214) involved in its regulation and by the observation that Brn-3b is down-regulated in RGC-5 cells, and presumably in primary RGCs as well, only when both mir-23 and mir-214 are transfected in RGC-5 cells. Intriguingly a similar microRNA expression profile, whereby mir-23 is expressed and mir-214 is not detectable, occurs in primary RGCs (Arora et al. 2007; Hackler et al. 2010) where Brn-3b is essential for their survival (Gan et al. 1996; Erkman et al. 2000) and in breast cancers and a the breast-cancer derived MCF-7 cell line (Volinia et al. 2006; Kovalchuk et al. 2008), in which Brn-3b has been shown to have a role in cell proliferation and invasiveness (Dennis et al. 2001).

The last chapter of the results initially assessed whether the phenomenon of PTGR plays a role in the differentiation of retinal ganglion cells. This was investigated by manipulating the expression of Dicer and by analyzing its effect on the levels of several RGC markers such as MAP2C, involved in neurite outgrowth (Lieven et al. 2007), and of Brn-3b and cATH5, two transcription factors involved in RGC development (Liu et al. 2001; Ma et al. 2004). These experiments drew a correlation between the expression of Dicer and the levels of RGC markers. Via a still unknown mechanism, increased Dicer levels are correspondingly followed by an increase in MAP2C and Brn-3b levels and a concomitantly decrease of cATH5 levels. This could be

due to a modification in the production of specific microRNAs targeting these RGC markers. Due to the focus of this thesis on the post-transcriptional regulation of Brn-3b, the effect of manipulating Dicer levels on RGC markers has not been followed any further. In order to analyze this aspect, RGC-5 cells could be transfected with a Dicer over-expression vector or conversely with a vector encoding for specific shRNA which suppress its expression and subsequently perform a profiling of the microRNA population resulting from this manipulation. The identified microRNAs could be then matched to their putative targets *via* an *in silico* analysis which could suggest whether any of the microRNAs target cATH5 and other RGC markers

The data presented in this thesis indicate that Brn-3b, via sequences contained in its 3'UTR seems to be subjected to regulation by specific microRNAs in some but not all the cell lines tested. As a working hypothesis, due to its essential role in the survival of some cell lines, Brn-3b might be protected from degradation by silencing the expression of mir-214 and by allowing the degradation of Brn-3b only when both mir-23 and mir-214 are expressed. Although at the present moment no other microRNAs targeting Brn-3b have been identified, their existence cannot be totally excluded. Very often the understanding of biological phenomena triggers more questions than the answers it provides, and thus several issues concerning the regulation of Brn-3b in RGCs remain yet to be addressed.

Firstly it would be interesting to understand whether Brn-3b plays a direct role in the transcriptional regulation of mir-214 and/or mir-23 promoter. This would open an intriguing scenario whereby Brn-3b regulates the expression of a microRNA that has a causative role in its degradation. This could be performed by subcloning the mir-23 and

mir-214 promoters upstream of a luciferase reporter gene, transfect the resulting construct in different cell lines and evaluating whether Brn-3b has any role in their regulation.

Secondly, it would be interesting to subclone the 3'UTR of chick Brn-3b and evaluate whether it is subjected to a similar regulation by microRNAs in primary RGCs. Although murine mir-23 and mir-214 do not have a conserved binding site on the chick 3'UTR of Brn-3b, it is possible that other yet to be identified microRNAs, still modulate the expression of Brn-3b in RGCs. This should be associated to an evaluation of mir-23 and mir-214 expression during chick development.

Since Dicer knock out mice die at very early stages of development (Bernstein et al. 2003) it would be intriguing to explore whether, besides being an essential enzyme in the production of mature microRNAs, Dicer is involved in other still undisclosed essential functions. In order to assess this aspect in RGC development it will be important to separate the role that Dicer has in the production of microRNAs from any other essential function it may have in neuronal differentiation. This could be accomplished by silencing Dicer function in RGCs and by co-injecting the same neurons with microRNAs extracted from developing RGCs. If Dicer is only essential to produce microRNAs and these molecules play a direct role in the differentiation of RGCs, neurons transfected with microRNAs extracted from differentiating RGCs, should be complemented for the deficit of Dicer and will be able to grow axonal projections as the wild type does. If Dicer also plays an undisclosed essential role in neuronal differentiation, the phenotype will not be rescued and neurons will not be able to differentiate and will die.

Lastly, I would like to report an anecdote related to the approach I would like to have in regard of natural phenomena in my scientific career.

When I was studying medicine I was taught that stomach ulcers were caused, among other reasons, by abuse of coffee, cigarettes and by stress. As a student, I had always wondered what “stress” was and why, in the medical environment, it was so often used to explain so different diseases and states of morbidity.

Eventually, Dr Barry J. Marshall and Dr J. Robin Warren discovered that the “stress” causing stomach ulcer was actually a bacterium, *Helicobacter pilori* (Marshall et al. 1984). Thanks to their unorthodox and incredibly open-minded view they broke a dogma and gave a real explanation to the cause of stomach ulcer, at least until their discovery will be superseded/incorporated by new knowledge.

The history of science is full of examples like these but somehow they get forgotten, new dogmas arise and new people break them. We just have to remember that there are no such things as absolute truths, especially in science, and we have to draw on our absolute ignorance if we want to be able to invent new wheels.

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